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Genetic Relatedness of *Cryptococcus neoformans* Clinical Isolates Grouped with the Repetitive DNA Probe CNRE-1

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Cryptococcus neoformans **isolates from eight patients with cryptococcal infection were previously assigned into three groups on the basis of repetitive DNA probe (CNRE-1) restriction fragment length polymorphisms. These groups accounted for a disproportionate number of recent clinical isolates in New York City. To further examine the genetic relatedness of isolates within and across CNRE-1 groups, the DNA sequence of the 779-base *URA5* gene from each strain was amplified and sequenced. The number of nucleotide differences in pairwise comparisons ranged from 0 to 30 (0 to 3% of the total sequence). Most of the nucleotide differences occurred in the third codon position or in introns. Pairwise comparisons revealed average nucleotide differences within a CNRE-1 group of 4.8 ± 2.6 ($n = 8$) and between CNRE-1 groups of 21.9 ± 7.0 ($n = 20$) ($P < 0.001$). Analysis of *URA5* sequences defined three groups that were congruent with those defined by CNRE-1 restriction fragment length polymorphisms. PCR amplification of an rDNA intergenic spacer revealed conservation of the intergenic spacer length within groups. Electrophoretic karyotyping did not distinguish between two isolates in each of two CNRE-1 groups. DNA from all isolates studied hybridized to an α mating type-specific probe. We interpret these results as suggesting a clonal population structure for some pathogenic isolates of *C. neoformans* in New York City.**

Cryptococcus neoformans is a yeast-like basidiomycete that causes life-threatening infections in 6 to 8% of patients with AIDS (3). In New York City there were more than 1,200 clinical infections in 1991 (3). Clinical and environmental isolates from New York City exhibit extensive genetic diversity such that the majority of isolates can be distinguished by DNA typing methods (2, 5). Several studies have documented extensive genetic diversity among *C. neoformans* isolates by various DNA typing methods including restriction fragment length polymorphisms (RFLPs) (2, 4, 13, 14, 18, 19), electrophoretic karyotyping (12), allele sequencing (2), and random amplified polymorphic DNA analysis (10). Even within restricted geographic areas *C. neoformans* isolates are not genetically homogeneous. The existence of this variation is potentially important since genetically distinct strains may differ in their virulence and their response to therapy. Genetic variation is also an important consideration in the design of vaccines and immunotherapy of cryptococcosis.

CNRE-1 is a dispersed repetitive sequence present in 10 to 20 copies in *C. neoformans* and is found in all *C. neoformans* chromosomes (14, 16). CNRE-1 RFLPs have been used to study genetic diversity (14), the pathogenesis of persistent infection (15), and the relationship of environmental (pigeon excreta) and clinical isolates (4). The origin of distinct CNRE-1 patterns is not known. RFLP differences may arise from chromosomal rearrangements, sexual recombination and/or reassortment, or genetic drift. In a previous study of recent isolates from New York City, 17 clinical isolates exhibited 12 different CNRE-1 RFLP patterns, with three RFLP patterns (patterns *vi*, *vii*, and *viii*) accounting for 45% of the isolates (4). These isolates were all *C. neoformans* var. *neoformans* and had been recovered from patients with AIDS at one New York City hospital during a single calendar year (4). The predominance of a few CNRE-1 patterns among clinical isolates could represent the increased virulence of specific *C. neoformans* clones for human infection. However, an understanding of the significance of three predominant CNRE-1 patterns among isolates from these patients requires an appreciation of the level of genetic relatedness among isolates with identical CNRE-1 patterns, as well as between isolates with different CNRE-1 patterns.

The study described in this report investigated these issues by first comparing CNRE-1-based groupings with those obtained by several other DNA typing methods. Second, these results were compared with those obtained by groupings of isolates based on the degree of similarity of the *URA5* allele sequences as a reference standard.

MATERIALS AND METHODS

C. neoformans **isolates.** Isolates J10, J15, J16, J17, J18, J19, J20, J21, J22, J24, J25, and J26 have been described previously (4). The designation J denotes the origin from the Jacobi Hospital of the Bronx Municipal Hospital Center (Bronx, N.Y.). Strains 34873 (B-3501) and 34874 (B-3502) were obtained from the American Type Culture Collection (Rockville, Md.) and were used as reference strains for mating type α and **a**, respectively. Isolates were maintained at 4°C on Sabouraud dextrose agar.

DNA sequence determination. *C. neoformans* DNA was isolated as described previously (4). The *URA5* gene (coding for orotidine monophosphate pyrophosphorylase; OMPPase) (6) was amplified from genomic DNA by PCR with the oligonucleotides TTAAGACCTCTGAACACC and ATGTCCTCCCAAGCC CTC as described previously (2). PCR products were analyzed by agarose gel electrophoresis and were identified as *URA5* DNA by hybridization with the [γ-^{32}P]dATP-labelled oligonucleotide TCGTCCCTTGAGTGGCGC complementary to an internal *URA5* sequence by Southern blotting (see below). *URA5*-amplified DNA was ligated to the pCR1000 vector of the TA cloning system (Invitrogen, San Diego, Calif.), and *Escherichia coli* transformants were selected on plates containing 50 μg of kanamycin per ml. Plasmid DNA was purified by using Maxi-Prep columns (Qiagen, Chatsworth, Calif.), and the insert was se-

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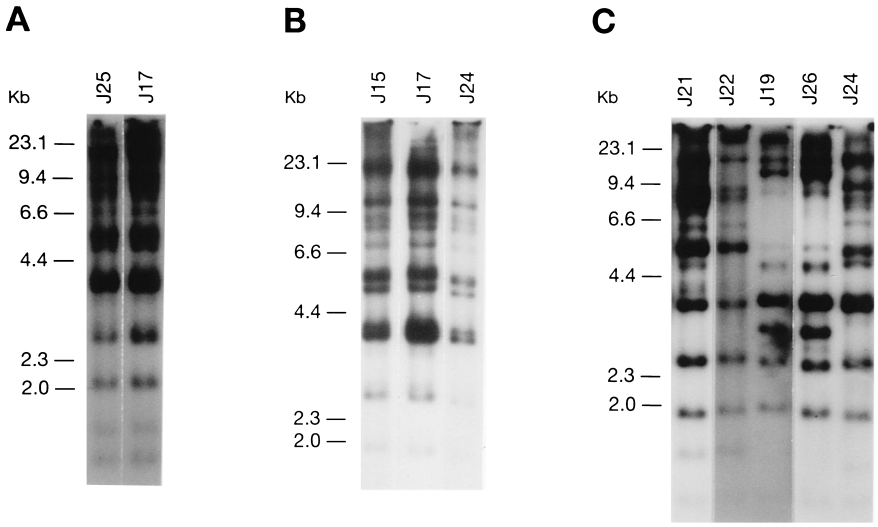

FIG. 1. Southern blot autoradiogram illustrating RFLPs for the three CNRE-1 groups. (A, B, and C) Composite photographs from single blots used to illustrate the RFLP patterns. (C) A darker exposure of the J19 and J22 RFLPs was used to enhance the contrast of the autoradiography bands.

quenced by the dideoxynucleotide-chain termination method with the Sequenase version 2.0 kit (United States Biochemicals, Cleveland, Ohio). Sequencing was done with the amplification oligonucleotides described earlier (2).

rDNA IGS amplification. The rDNA intergenic spacer (IGS) between the 23S-like and 16S-like ribosomal DNA (rDNA) genes was amplified with the primers 5′-GAGGCTAGAGGTGCCAG and 5′-ATTCGAGAAGTTATTATG as described previously (7). Amplification conditions were 1 min at 94°C, 1 min at 52°C, and 3 min at 72°C for 30 cycles in buffer with 2.0 to 2.5 mM $MgCl_2$.

Southern blot analysis. Mating type determination was done by probing Southern blots with mating type α-specific sequences (MATα probe) (11). The MATα probe was made by PCR amplification of genomic DNA from strain 34873 on the basis of the nucleotide sequence of the pheromone gene (11). The primers were 5′-CCAGCAACCAACCATCGC and 5′-GCGACTAACACGGC GGTC. PCR conditions were 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C for 30 cycles in a buffer with 1.5 mM $MgCl_2$. The reaction products were separated on a 4% Nusieve agarose gel (FMC, Rockland, Maine), and the 192-bp band was excised and purified with QIAEX (Qiagen). The fragment was reamplified, and its identity was confirmed by cycle sequencing. *Kpn*I digests of cryptococcal DNA were separated on 0.8 to 1.0% agarose, transferred to nylon membranes, and hybridized to ^{32}P-labelled α probe. α probe ^{32}P-labelling was done with [^{32}P]dCTP by using random hexamer primers. Hybridization conditions were 65°C in a solution of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 7% sodium dodecyl sulfate, 10× Denhardt's reagent, and 10% dextran sulfate in 0.020 M sodium phosphate (pH 7.2). The restriction enzymes, nylon membranes, and random priming kit were obtained from Boehringer Mannheim (Indianapolis, Ind.). CNRE-1 RFLP analysis was done exactly as described previously (5).

Electrophoretic karyotyping. Chromosomal DNA plugs were prepared from *C. neoformans* cells grown at 30°C in Sabouraud dextrose broth. Protoplasts were generated by incubation of cells in 1.0 M sorbitol–0.1 M sodium citrate (pH 6) with 10 mg of NovoZym 234 (Novo Biolabs, Bagsvaerd, Denmark) per ml for 0.5 to 2 h at 30°C. Protoplasts (4×10^8 to 6×10^8/ml) were mixed with 2% Pulse Field Certified Agarose (Bio-Rad, Richmond, Calif.) for a final agarose concentration of 0.75%, and the plugs were incubated overnight at 37°C in 1 mg of proteinase K (Boehringer Mannheim) per ml–1% sarcosine–0.1 M EDTA in 0.010 M sodium citrate (pH 8.0). The plugs were then washed four times in 50 ml of wash buffer (0.05 M EDTA, 0.020 M Tris-HCl [pH 8.0]) by incubating them at room temperature for 1 h per wash. After a final wash with 0.1× wash buffer for 1 h, the plugs were stored at 4°C until they were used. For chromosome separation the plugs were inserted into a 1.0% gel (12 by 14 cm), and electrophoresis was done in a CHEF DRIII variable-angle pulse-field electrophoresis system (Bio-Rad) in 0.5× TBE (Tris-borate-EDTA) buffer at 14°C. The system was equipped with a Cooling Module (Bio-Rad) for constant temperature control. Electrophoretic conditions were 60- to 120-s switch time ramp at 6.0 V/cm for 24 h at an included angle of 120°. The gels were stained with ethidium bromide and photographed.

DNA sequence analysis. *URA5* sequences were compared by parsimony and maximum likelihood methods by using the DNAPENNY (branch and bound parsimony) and DNAML (maximum likelihood) programs in PHYLIP version 3.5 (8). The data set consisted of the J isolates, B-3501 (6), and *C. neoformans* var. *gattii* (1). Only the 743-bp sequence between the amplification primers was analyzed. The data set was also bootstrapped by using the SEQBOOT and CONDENSE programs in PHYLIP version 3.5.

Nucleotide sequence accession numbers. The *URA5* DNA sequences of isolates J15, J17, J19, J21 (same as J22), J24, J25, and J26 have been deposited in the Genome Sequence Data Base (Santa Fe, N.M.) under accession numbers L38582, L38583, L38584, L38585, L38586, L38587, and L38588, respectively.

RESULTS

Isolates J15, J17, J19, J21, J22, J24, J25, and J26 had previously been assigned into three groups on the basis of CNRE-1 RFLP patterns, known as *vi* (J21, J22), *vii* (J19, J26), and *viii* (J15, J17, J24, J25) (4). The CNRE-1 RFLPs of these groups differed from each other in at least two bands (Fig. 1). The sequences of the *URA5* alleles are shown in Fig. 2. The group *vi* isolates were the only two that had identical sequences. The group *vii* isolates differed at three positions (all within introns). The group *viii* isolates differed at nine positions (six within introns). Variation in the *URA5* sequence within a CNRE-1 group was significantly less than variation between groups. The numbers of *URA5* base differences (average ± standard deviation) within and between groups were 4.8 ± 2.6 ($n = 8$) and 21.9 ± 7.03 ($n = 20$), respectively ($P < 0.001$, Student's *t*-test). The majority of base differences occurred in introns or the third positions of codons.

Parsimony analysis of the DNA sequences revealed a single most parsimonious tree that contained J21 and J22 on one branch, J19 and J26 on a second branch, and J15, J17, J24, and J25 on a third branch (Fig. 3). The last group was divided into two subbranches containing isolates J15 and J17 and isolates J24 and J25. An identical tree was obtained by maximum likelihood analysis (data not shown). Bootstrap analysis supported these three branches at a level of ≥86% (subbranches containing isolates J15, J17, J24, and J25 were present in 54 to 62% of the trees). Thus, differentiation of the eight isolates on the basis of the sequence of the *URA5* gene yielded the exact same groups as those determined by CNRE-1 RFLPs.

Differences in the predicted enzyme sequence (OMPPase, the product of the *URA5* gene) were consistent with those found in the parsimony analysis. The group *vii* isolates coded for identical OMPPases that differed from J21 at codons 8 and 57. The group *viii* isolates differed from J21 at codons 8 and 11.

```
J21,J22   ATG TCC TCC CAA GCC CTC GAC TTC GCC AAA GTT GCC TTC ATC GAG GCT GCC ATC GAA CAT GGC GTG CTT CTT TTC GGC AAC TTT ACC TTG AAG TCC GGC CGgtgagccatattg
J19       --- --- --- --- --- --- --- -C- --- --- --- --- --t --t --a --- --a --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -------------c-
J26       --- --- --- --- --- --- --- -C- --- --- --- --- --t --t --a --- --a --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---------------
J15       --- --- --- --- --- --- --- -C- --- --- A-- --- --- --t --a --- --a --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---------------
J17       --- --- --- --- --- --- --- -C- --- --- A-- --- --- --t --a --- --a --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---------------
J24       --- --- --- --- --- --- --- -C- --- --- A-- --- --- --t --a --- --a --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---------------
J25       --- --- --- --- --- --- --- -C- --- --- A-- --- --- --t --a --- --a --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---------------

J21,J22   cagcgcttcacagtccaatcgaatctgacatgtgttcagT   CAA TCC CCT TAC TTC TTC AAT GCC GGT CTC CTT TAC TCT TCA TCG CTT CTC TCA ACT ACC GCT CAG GCT TAC GCC AAG
J19       -------------------------------a---c----   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --a --- --- --- --- --- --- --- -G- --- --- ---
J26       -------------------------------a--------   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --a --- --- --- --- --- --- --- -G- --- --- ---
J15       -----a----t-----------------------------   --- --- --- --- --- --- --- --- --- --- --- --t --- --- --a --- --- --- --- --t --c --- --- --- --- ---
J17       --ca-g----t-------n---------------------   --- --- --- --- --- --- --- --- --- --- --- --t --- --- --a --- --- --- --- --t --c --- --- --- --- ---
J24       --ca-t----t-----------------------------   --- --- --- --- --- --- --- --- --- --- --- --t --- --- --a --- --- --- --- --t --c --- --- --- --- ---
J25       ----------t-----------------------------   --- --- --- --- --- --- --- --- --- --- --- --t --- --- --a --- --- --- --- --N --c --- --- --- --- ---

J21,J22   GTA CTT TCC TCT TCT AGG ATT CCT GAC TTT GAC GTC CTC TTC GGC CCA GCT TAC AAG GGT ATC TCC TTG GCT GCT GTC TCC GCT GTA AGC CTT TAT CAG CAA ACC GGC AAA
J19       --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --g --- --- ---
J26       --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --g --- --- ---
J15       --- --- --- --- --- --- --- --c --- --- --- --- --- --t --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --g --- --- ---
J17       --- --- --- --- --- --- --- --c --- --- --- --- --- --t --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --g --- --- ---
J24       --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --g --- --- ---
J25       --- --- --- --- --- --- --- --c A-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --g --- --- ---

J21,J22   GAT ATC GGT TAC TGC TAC AAC AGG AAG GAG AAG AAG GAC gtgagtctgtcctaaccagtgcgacagcgatgagctcataagccagtag   CAC GGC GAG GGC GGT ACT ATG GTC GGT GCG CCT
J19       --- --- --- --- --- --- --- --- --- --- --- --- --- --a------------------------------a---------------   --t --- --- --- --- --- --- --- --- --a ---
J26       --- --- --- --- --- --- --- --- --- --- --- --- --- --a----------------------------------------------   --t --- --- --- --- --- --- --- --- --a ---
J15       --- --- --- --- --- --- --- --- --- --- --- --- --- --a-------------------------------------------c--   --t --- --- --- --- --- --- --- --- --a ---
J17       --- --- --- --- --- --- --- --- --- --- --- --- --- --a-------------------------------------------c--   --t --- --- --- --- --- --- --- --- --a ---
J24       --- --- --- --- --- --- --- --- --- --- --- --- --- --a----------------------t-----------------------   --t --- --- --- --- --- --- --- --- --a ---
J25       --- --- --- --- --- --- --- --- --- --- --- --- --- --a---------------------g------------------------   --t --- --- --- --- --- --- --- --- --a ---

J21,J22   CTC AAG GGA CGA ATC GTC ATC ATC GAC GAT GTT CTC ACC TCT GGC AAG GCC ATC CGT GAA GCT ATT GAC ATT CTC AAG GCC TCC CCT GAA GCG AAG CTT GTT GGA ATT GTC
J19       --- --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- ---
J26       --- --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- ---
J15       --- --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- --- --t --- --- --- --- --c --- --- --- ---
J17       --- --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- --- --t --- --- --- --- --c --- --- --- ---
J24       --- --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- --- --t --- --- --- --- --c --- --- --- ---
J25       --- --- --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- --- --t --- --- --- --- --c --- --- --- ---

J21,J22   CAG CTT GTC GAC AGA CAA GAG AAA GGC CAG AGC GGT AGC GGC AAG AGT ACC GTA CAG GAG GTT GAG GAA GAG TTC GGT GTG CCT GTC GAG CCT ATC ATT GGT TTG GAT GAT
J19       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- ---
J26       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- ---
J15       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- ---
J17       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- ---
J24       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- ---
J25       --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --c --- --- --- --- --- --- --- --- ---

J21,J22   ATT GTG AAG TAC TTA GAA AGC TCC GGC AAG TGG GAA AAG GAG CTG CAA GAG GTC AGG AAG TAC CGG GCG GAG TAC GGT GTT CAG AGG TCT TAA
J19       --c --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
J26       --c --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
J15       --c --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
J17       --c --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
J24       --c --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
J25       --c --- --- --- --- --- --- --t --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
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FIG. 2. Nucleotide sequence of the *URA5* gene from isolates J21, J22, J19, J26, J15, J17, J24, and J25. A hyphen indicates that the base is identical to that in J21 (and J22). An N indicates that the identity of the base is uncertain. Lowercase letters indicate synonymous substitutions. Uppercase letters indicate nonsynonymous substitutions. The four nonsynonymous substitutions occurred at codons 8, 11, 57, and 69 and resulted in amino acid changes (from the J21 sequence) of Phe→Ser, Val→Ile, Ala→Gly, and Asp→Asn, respectively. Since the sequence was obtained from DNA amplified with *URA5*-specific primers (sequence underlined), the first and last 18 bases in the sequence of each of the J isolates corresponds to the amplification primer.

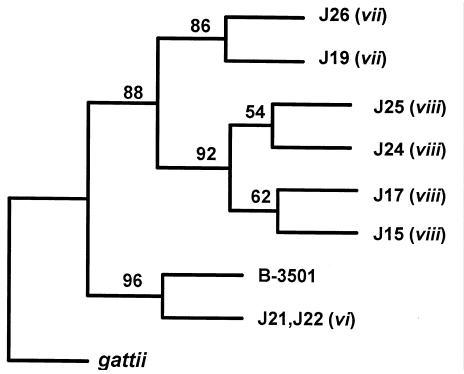

FIG. 3. Relationship of *C. neoformans* isolates derived from analysis of *URA5* sequence data. The phylogram shown is the strict consensus of the most parsimonious trees generated by the DNAPENNY program. The CNRE-1 groups are listed in parentheses after the name of each isolate. Numbers at the branch points indicate the percentage of times that the cluster consisting of the isolates to the right of the fork occurred among 200 bootstrap replications. *C. neoformans* var. *gattii* was designated the outgroup.

All four group *viii* isolates had identical coding sequences except for J25 which contained a nonsynonymous change at codon 69. This change was not confirmed and may represent a *Taq* polymerase error.

All isolates in groups *vi*, *vii*, and *viii* (except J15 which was not studied) had bands that hybridized to the MATα probe by Southern blotting and hence are presumed to be mating type α (Fig. 4). No bands were present in the negative control (mating type *a* strain ATCC B-3502). A band of approximately 10 to 12 kb was present in the positive control (α strain ATCC B-3501). Four RFLP patterns were observed in the three CNRE-1 groups. Group *vi* isolates J21 and J22 were not distinguishable by MATα locus RFLPs. RFLPs for group *vii* isolates J19 and J26 differed at two band positions. Group *viii* isolates J17, J24, and J25 were not distinguishable by MATα RFLPs. Four other isolates, J10, J16, J18, and J20 which differ in their CNRE-1 RFLPs from those for isolates in groups *vi*, *vii*, and *viii* (4), also differed by MATα RFLPs (Fig. 4).

The *C. neoformans* IGS is 2.4 kb, but the IGS lengths of strains can differ by 200 to 300 bp (7). The rRNA IGS DNAs from all strains except J15 were amplified and were analyzed by agarose gel electrophoresis (data not shown). The IGSs of group *vi* isolates were 100 to 200 bases smaller than those of

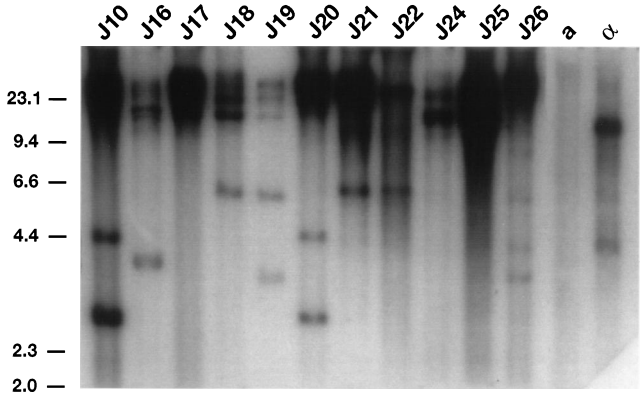

FIG. 4. Southern blot autoradiogram showing the hybridization of the MATα probe to *Kpn*I-digested DNA from the several J isolates and reference α and **a** mating types. The figure is a composite in which one empty lane between the J26 and **a** strain was removed.

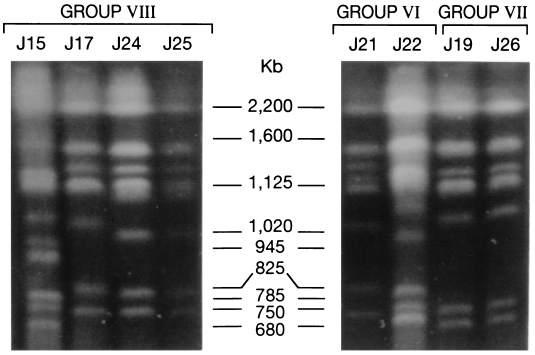

FIG. 5. Electrophoretic karyotypes of group *vi*, *vii*, and *viii* isolates. Group *vii* isolates J26 and J19 and group *viii* isolates J24 and J25 were not distinguishable by electrophoretic karyotypes.

isolates in groups *vii* and *viii*. Isolates within groups were not distinguishable by rRNA IGS length polymorphisms.

Electrophoretic karyotypes for isolates in groups *vi*, *vii*, and *viii* are shown in Fig. 5. Group *vi* isolates J21 and J22 had different electrophoretic karyotypes. Group *vii* isolates J19 and J26 were not distinguishable by electrophoretic karyotype. Among group *viii* isolates, isolates J24 and J25 were not distinguishable by electrophoretic karyotype, but their karyotypes differed from those of isolates J15 and J17. All strains had 10 to 12 chromosomes, consistent with the average number for *C. neoformans* (20).

DISCUSSION

In previous studies we showed that a large proportion of clinical and environmental isolates of *C. neoformans* could be distinguished by CNRE-1 RFLPs (4). A limitation of this methodology is its inability to estimate how genetically distinct each isolate with a different CNRE-1 pattern is. It was also not known whether strains with identical patterns were closely related. These issues are intertwined with questions concerning the extent of sexual reproduction among *C. neoformans* isolates in nature.

To investigate these issues we sequenced the *URA5* alleles of isolates with the same CNRE-1 pattern. The *URA5* gene was chosen for investigation because previous studies suggested that it is polymorphic (1, 2), found in one copy per genome (6), and essential for uracil synthesis (6). If *URA5* was highly polymorphic and sexual reproduction was common, then isolates with identical CNRE-1 patterns could contain different *URA5* alleles while isolates with different CNRE-1 patterns could have the same *URA5* allele. The current study reveals that even within a set of isolates that are restricted geographically and temporally, the *URA5* gene sequence was highly polymorphic. However, the *URA5* alleles were not distributed randomly. Grouping of alleles by parsimony analysis was exactly congruent with grouping of isolates by CNRE-1 pattern. This strongly suggests that in this population, isolates with identical CNRE-1 patterns have been genetically isolated from those in other groups. All of the URA5 sequence differences within a CNRE-1 group (with one possible exception) occurred within introns or were translationally silent third base changes and probably represent genetic drift. The observations that most isolates within groups share MATα RFLPs and rDNA IGSs of the same length are consistent with the idea of isolated populations.

A clonal population structure has tentatively been suggested

for *C. neoformans* on the basis of multilocus enzyme electrophoresis data (17). The preponderance of the α mating type in both clinical and environmental samples is also suggestive of a primarily asexual mode of reproduction (9). Although our results are consistent with a clonal population structure for these isolates, they do not exclude the possibility that the ancestral clones had a sexual origin. The unequal distribution of CNRE-1 groups among clinical isolates from New York City may be due to uncharacterized selective forces.

The proliferation of DNA typing schemes in recent years has revealed extensive genetic diversity among *C. neoformans* isolates. DNA typing is highly discriminatory, but isolates grouped by one DNA typing technique may be distinguishable by other techniques and vice versa. The variation in sensitivity among DNA typing methods and extensive genetic diversity among *C. neoformans* isolates raises the question of how best to define a "strain." At one extreme, defining strains by DNA sequence variation would result in assigning most isolates to different strains. By the *URA5* sequence criteria the eight isolates studied here would constitute seven strains. At the other extreme, defining strains on the basis of RFLPs would group strains that may differ at other loci. For example, the isolates assigned to the three CNRE-1 groups differ by *URA5* sequence (groups *vii* and *viii*) or electrophoretic karyotype (group *vi*). While *URA5* sequencing is clearly the most discriminatory of the typing methods investigated, the present study also confirms that the CNRE-1 probe remains a highly discriminatory typing tool. When *URA5* sequence data are used to construct isolate groups on the basis of the similarity of sequences, the resulting groups were exactly congruent with the CNRE-1-defined groups. Thus, CNRE-1 RFLPs provide a means of grouping isolates for comparison of phenotypic and genotypic traits and can be used to define strains.

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