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Molecular Epidemiology of Clinical *Cryptococcus neoformans* Strains from India

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Little is known about the molecular epidemiology of the human pathogenic fungus *Cryptococcus neoformans* in India, a country now in the midst of an epidemic of AIDS-related cryptococcosis. We studied 57 clinical isolates from several regions in India, of which 51 were *C. neoformans* var. *grubii*, 1 was *C. neoformans* var. *neoformans*, and 5 were *C. neoformans* var. *gattii*. This strain set included 18 additional sequential isolates from 14 patients. Strains were characterized phenotypically by measuring the polysaccharide capsule and by determining the MICs of standard antifungals. Molecular typing was performed by a PCR-based method using the minisatellite-specific core sequence (M13), by electrophoretic karyotyping, by restriction fragment length polymorphisms with the *C. neoformans* transposon 1 (TCN-1), and by *URA5* DNA sequence analysis. Overall, Indian isolates were less heterogeneous than isolates from other regions and included a subset that clustered into one group based on *URA5* DNA sequence analysis. In summary, our results demonstrate (i) differences in genetic diversity of *C. neoformans* isolates from India compared to isolates from other regions in the world; (ii) that DNA typing with the TCN-1 probe can adequately distinguish *C. neoformans* var. *grubii* strains; (iii) that TCN-1 sequences are absent in many *C. neoformans* var. *gattii* strains, supporting previous studies indicating that these strains have a limited geographical dispersal; and (iv) that human cryptococcal infection can be associated with microevolution of the infecting strain and by simultaneous coinfection with two distinct *C. neoformans* strains.

Cryptococcus neoformans is an encapsulated yeast which causes life-threatening infections in approximately 2 to 3% of patients with AIDS in the United States (42) and in up to 40% of AIDS patients in Africa (www.who.int/hiv/; 2004 data). Meningoencephalitis is the most common and serious clinical manifestation of *C. neoformans* infection and can be refractory to antifungal therapy (50). In the absence of immune reconstitution with antiretroviral therapy, the initial presentation requires lifelong suppressive antifungal treatment to reduce the likelihood of recurrent cryptococcosis. Genetic differences among *C. neoformans* strains have been detected by several typing methods, including restriction fragment length polymorphism (RFLP) analysis (18, 20), electrophoretic karyotyping (7, 20, 24), allele sequencing (15), multilocus enzyme electrophoresis (9, 10), and random amplified polymorphic DNA analysis (6, 8, 9). Understanding the genetic variation of a pathogen population is important because it could translate into differences in the host's immunogenic response and therefore become a major consideration in vaccine design. In addition, genetic variation could affect the function of important genes and thereby affect virulence. Furthermore, knowledge of genetic variation is essential for understanding the population structure and evolution of a microorganism (55).

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The tropical climate of the Indian subcontinent offers a suitable environment for *Cryptococcus neoformans*, and the onslaught of the AIDS pandemic since the early 1990s has led to a sharp increase in the number of reported cases of cryptococcosis in the past decade (3–5). In India, this number is likely to be an underestimate because cryptococcosis is not a reportable disease. Furthermore, since India's population exceeds one billion and because aggressive retroviral therapy may not be available for all affected individuals, the absolute numbers of infected patients at risk for AIDS-related cryptococcosis in the near future are likely to be very high. The wide variety of presentations of the disease seen in India suggests the possibility of strain variation. In a finding similar to that of an Australian survey (17), many of the Indian cases occur in patients with no obvious impairment of the immune system (2). Furthermore, the distribution of serotypes in Indian clinical isolates appears to be different from that found in other regions, with the majority of the Indian isolates being serotype A and a significant minority being serotype B (2). Since India, the most densely populated country in the world, is facing a significant increase in AIDS cases and cryptococossis, undertaking a detailed investigation of the *C. neoformans* strains is warranted. The current study was undertaken to investigate the molecular epidemiology of *C. neoformans* infection in India and yields important results about *C. neoformans* strains from this part of the world.

MATERIALS AND METHODS

Cryptococcal isolates. A total of 57 clinical Indian *C. neoformans* isolates, which were derived from a total of 39 patients, were included in the study. The isolates originated either from patients admitted to All India Institute of Medical Sciences (AIIMS) in Delhi or were sent for identification to AIIMS from different cities in India (this includes one isolate that was sent from Nepal). All clinical isolates were obtained over a 1-year period. All clinical isolates were identified as *C. neoformans* by brown colony color in bird seed agar, positive urease test, sugar assimilation test, and ability to grow at 37°C. Isolates that produced black colonies in L–3,4-dihydroxyphenylalaline (L-DOPA) agar were identified as *C. neoformans* var. *neoformans* or var. *grubii* on the basis of no color change on canavanine-glycine-bromothymol blue agar (25). Mating type was determined by amplifying Matα-specific sequences as described previously (28).

C. neoformans strains H99 (serotype A), 24064 (serotype A), 24067 (serotype D), J11 (serotype A), B3501 (serotype D), JEC21 (serotype D), and SB4 (serotype A) were used as reference strains for RFLP typing. For RFLP typing and for *URA5* sequence comparison of *C. neoformans* var. *gattii* strains, DNA was isolated from 12 environmental isolates and 1 clinical isolate from Australia (AS2559, AS2557, AS2554, AS2552, AS1337, AS2553, AS2561, AS2551, AS2555, AS2556, AS2558, AS2560, and AS2562), 5 clinical isolates from Canada (CN2615, CN2611, CN2613, CN2618, and CN2617), and 1 clinical serotype C isolate from New York (NY1343C) (generous gift from Tom Mitchell). Furthermore, the *URA5* DNA sequence was downloaded (see below) for seven New York City isolates (J15, J17, J19, J21, J24, J25, and J28) and on five Brazilian strains (BZ1, BZ2, BZ3, BZ4, and BZ5), of which all but the J21 strain (serotype D) were serotype A strains.

Capsule measurement and staining. Capsule size of in vitro-grown yeast cells was examined at a magnification ×100 under oil in an India ink suspension. The distance from the cell wall to the outer margin capsule and the cell diameter (not including the capsule) was measured using an eyepiece grid with a resolution of 0.5 μm. The polysaccharide capsule of unfixed yeast cells was stained with a monoclonal antibody (MAb) to glucuronoxylomannan 12A1 and an isotype-specific fluorescein-labeled secondary MAb (51).

In vitro antifungal susceptibility testing. The MICs for amphotericin B and fluconazole were determined by CLSI (formerly NCCLS) M27-A methodology (48, 49). As a quality control measure, the results were considered valid only when the MICs of the quality control isolates fell within the prespecified ranges: *Candida parapsilosis* ATCC 22019 (fluconazole, 2 to 8 mg/liter) and *Candida krusei* ATCC 6258 (fluconazole, 16 to 64 mg/liter). Prior to testing, each isolate was subcultured at least twice on Sabouraud's dextrose agar plates to ensure purity and optimal growth. Suspensions were prepared in 0.85% saline to achieve 1 McFarland standard adjusted by spectrophotometer measurement. A working solution was prepared by a 1:1,000 dilution in RPMI 1640 buffered with morpholinepropanesulfonic acid. The working cell suspension (0.9 ml) was added to 0.1 ml of a 10-fold dilution of drug ranging from 1.25 to 640 μg/ml and 0.03 to 40 μg/ml for fluconazole and amphotericin B, respectively. The tubes were incubated at 30°C for 48 h. The MIC was determined as the lowest concentration that produced at least 80% inhibition compared to the growth of the control tube for fluconazole and the absence of visible growth in the case of amphotericin B. The 80% inhibition standard for fluconazole was prepared by diluting 0.2 ml of drug-free control growth with 0.8 ml of RPMI medium. The turbidity was then compared both visually and spectrophotometrically.

Genomic DNA isolation. *C. neoformans* DNA was isolated by using a modification of an existing protocol (22). Briefly, protoplasts were generated by incubating cells in 10 mg/ml lysing enzyme (from *Trichoderma harzianum*; Sigma Aldrich, St. Louis, MO) in 1.0 M sorbitol–0.1 M sodium citrate (pH 5) for 1 to 3 h at 30°C. Protoplasts were lysed in a solution of 5 mM EDTA, 10 mM sodium citrate, and 1% sarcosine. After 40 min at 65°C, the lysate was extracted twice with buffer-equilibrated phenol and once with chloroform. DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethyl alcohol and was resuspended in 0.01 M Tris-HCl—0.01 M EDTA (pH 8.1).

RAPD analysis. Randomly amplified polymorphic DNA (RAPD) analysis was done by a PCR-based method using the minisatellite-specific core sequence of the wild-type phage M13 (5′GAGGGTGGCGGTTCT 3′) (40) as a single primer in the PCR. The amplification reactions were performed in a volume of 50 μl containing 25 ng high-molecular-weight genomic DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl, 0.2 mM (each) of dATP, dCTP, dGTP, and dTTP, 3 mM magnesium acetate, 30 ng primer, and 2.5 U Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA). PCR was performed for 35 cycles in a Perkin-Elmer thermal cycler (model 480) with 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and a 1-min extension at 72°C, followed by a final extension cycle for 6 min at 72°C. Amplification products were removed, concentrated to approximately 20 μl, separated by electrophoresis on 1.4% agarose gels (stained with ethidium bromide, 10 mg/ml stock) in 1× Tris-borate-EDTA buffer at 60 V for 14 cm, and visualized under UV light. All visible bands were included in the analysis, independent of their intensity. Band patterns fell into eight groups. Strains were classified as follows: groups VNI and VNII (serotype A), VNIII (serotype AD), VN IV (serotype D), and VG I, VG II, VG III, and VG IV (serotypes B and C) (40).

RFLP analysis. All isolates were typed by Southern blot analysis with a *C. neoformans*-specific transposon sequence (TCN-1) (33). Briefly, genomic DNA was extracted from protoplasts. Fifty micrograms of genomic DNA was digested with 50 U of EcoRI (Invitrogen) for 4 h at 37°C. The resulting fragments were resolved on a 1% agarose gel in 0.5× Tris-acetate-EDTA buffer at 32 V for 24 h and transferred to a positively charged nylon membrane. Hybridization with the labeled TCN probe was done in a solution of denatured salmon sperm DNA (0.75 mg/ml) in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 7% sodium dodecyl sulfate (SDS), 10× Denhardt's reagent, and 5% dextran sulfate in 0.02 M sodium phosphate buffer (pH 7.2) at 65°C. Filters were washed with wash solution 1 (3× SSC, 5% SDS, 10× Denhardt's reagent) and then wash solution 2 (1× SSC, 1% SDS) at 65°C. The TCN-1 probe was generated by amplifying a 634-bp fragment from strain H99 with specific primers (TCN1.F.2 [5′-TCATGTCAGGTCCTTCCACTCGTAG-3′]) and (TCN1.R.2 [5′-CAT AAACTTGGGCTGGGGATCG-3′]). Sequence comparison of the TCN amplicon from H99 (serotype A), B3501 (serotype D), and NP-1 (serotype B) demonstrated 94% identical sequence for serotypes A and B and 96% for serotypes A and D. The TCN amplicon was purified, cloned, and labeled with [α-^{32}P]dCTP using the High Prime DNA labeling kit (Boehringer, Mannheim, Germany). The bands were visualized by autoradiography. The RFLP patterns were normalized by equating the 1-kb DNA molecular weight markers (Gibco).

URA5 gene sequencing. The 724-bp (serotypes A and D) and 447-bp (serotype B) sequence of the *URA5* gene (coding for orotidine monophosphate pyrophosphorylase) was amplified from genomic DNA by PCR with oligonucleotides (for serotypes A and D, 5′-TTAAGACCTCTGAACACC-3′ and 5′-ATGTCCTCC CAAGCCCTC-3′; for serotype B, 5′-CCGGTCTCCTTTATTCCTCA-3′ and 5′-AAACCAATRATAGGCTCGAC-3′) as described previously (21) and sequenced in the DNA sequencing facility at Albert Einstein College of Medicine. Samples were analyzed by fluorescent cycle sequencing with dye-labeled primers. The *URA5* sequences obtained from the reference strains (17 from *C. neoformans* var. *gattii* and 28 from *C. neoformans* var. *neoformans*) were compared with *URA5* sequences downloaded from GenBank (GB). These include *URA5* sequences of seven New York City clinical isolates (GB no. L38582 to L38588, respectively), 5 Brazilian clinical isolates (GB no. U67723 to U67727, respectively), B-3501 (GB no. M34606), ATCC 24064 (GB no. AF032432), and a *C. neoformans* var. *gattii* reference strain (GB no. M93026).

Chromosomal DNA isolation and electrophoretic karyotyping. Karyotype analysis was done by contour-clamped homogeneous electrophoresis (CHEF). Chromosomal DNA plugs were prepared from cultures derived from single colonies as described previously (24). Protoplasts were generated by digesting cells with a variety of concentrations of lysing enzyme per ml (from *Trichoderma harzianum*; Sigma Aldrich) in 1.0 M sorbitol–0.1 M sodium citrate (pH 5) for 1 to 3 h at 30°C. Proper protoplast formation was verified by phase microscopy prior to proceeding with the isolation of chromosomal DNA. Protoplast-agarose plugs were made by mixing a protoplast suspension (4×10^8 to 6×10^8/ml) with 2% low-melting-temperature agarose solution (Bio-Rad, Richmond, CA) to yield a final agarose concentration of 0.66%. Plugs were allowed to harden at 4°C and were then incubated overnight at 50°C in a solution of 1 mg/ml of proteinase K (Boehringer Mannheim), 1% sarcosine, and 0.1 M EDTA–0.01 M sodium citrate (pH 8.0). The plugs were then washed four times in 50 ml of wash buffer (0.05 M EDTA, 0.02 M Tris-HCl [pH 8.0]). After a final wash in 0.1× wash buffer, the plugs were stored at 4°C until used. The plugs were inserted into a 1% pulse field certified agarose gel (Bio-Rad), and electrophoresis was done in a CHEF DRIII variable-angle pulse-field electrophoresis system (Bio-Rad) in 0.5% Tris-borate-EDTA at 12°C. The system was equipped with a cooling module (Bio-Rad) for constant temperature control. The electrophoresis conditions were programmed in two sequential blocks. First a switch time of 90 s was applied for 9 h and then a switch time of 120 s and 360 s for 63 h. Both blocks were run at 3.5 V/cm at an angle of 115°. Gels were stained with ethidium bromide and photographed.

Data analysis. The karyotype and TCN-1 RFLP patterns of all strains were compared by visual inspection. The RFLP patterns were normalized by equating the 1-kb and 100-bp molecular weight markers (Boehringer Mannheim). Similarly, *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad, Richmond, CA) was used in a lane as a reference to normalize the CHEF patterns. Patterns were

TABLE 1. Characteristics of *C. neoformans* (serotype A and D) strains from India

Strain	Age (yr)[a]	Source of sample	Clinical presentation	HIV status	URA5 cluster	Pattern determined by:		MIC FLU[i]	Mating type
						CHEF	RFLP		
I14	50	Sputum	SKIN	Negative	1	IV	I	24	α
I19	42	CSF	CME[b]	Negative	2	SM-IV	SM-M	32	α
						MC-III	MC-N	16	α
I31	46	CSF	HA[c]	Negative	2	I	B	8	α
I47	36	Sputum	COUGH	Positive		IV	H	6	**a**
I48	7	Blood	FUO[d]	Positive	2	III	B	8	α
I51	25	CSF	CME	Negative		III	B	4	α
I55	28	CSF	FUO	Positive		III	B	32	α
I57	45	CSF	HA	Positive	1	VII	G	8	α
I58	30	CSF	HA	Positive	2	VIII	G	64	α
I61	75	CSF	AS[e]	Negative	2	III	A	16	α
I62	24	CSF	CME	Positive		I	B	16	α
I64	23	CSF	CME	Positive	2	III	B	32	α
I65	18	CSF	CME	Negative		V	B	8	α
I66	24	CSF	FUO, HA	Negative		IX	A	32	α
I68	27	CSF	CME	Positive		XIII	L	32	α
I70	43	CSF	FUO	Negative	2	XIV	B	8	α
I71	44	CSF	CME	Negative	2	XIII	B	8	α
I72	22	CSF	CME	Positive		XII	B	4	α
I82	63	Blood	FUO	Negative	2	XV	B	16	α
I88	33	CSF	HA, AS	Positive		ND[h]	ND	2	α
I91	32	CSF	CME	Negative		III	B	4	α
I107	65	CSF	CME	Negative		I	C	8	α
I108	28	CSF	CME	Negative	1	III	B	8	α
I113	42	CSF	FUO	Positive	1	I	O	32	α
I114[f]	26	CSF	CME	Negative	1	X	O	0.25	α
I116	35	CSF	COUGH	Positive	1	X	O	32	α
I118	42	CSF	FUO	Positive	2	III	F	32	α
I119	35	CSF	CME	Negative	2	III	B	16	α
I120	28	CSF	CME	Positive		VII	B	8	α
I126	35	CSF	FUO, AS	Negative		III	E	8	α
ISG12	NA[g]	CSF	CME	NA		XI	K	4	**a**
ISG7	NA	CSF	CME	NA		XI	N	4	α
ISG2	NA	CSF	CME	NA	2	XI	N	4	α
IJP1	NA	NA	NA	NA	2	III	B	4	**a**

[a] Age of patient.
[b] CME, chronic meningoencephalitis.
[c] HA, headache.
[d] FUO, fever of unknown origin.
[e] AS, altered sensorium.
[f] Serotype D strain.
[g] NA, not analyzed.
[h] ND, not determined.
[i] MIC of fluconazole.

considered identical if all bands matched exactly. Patterns that differed by only one band were identified as subtypes. Isolates that differed from the others by at least two bands were assigned to different patterns. Similarity coefficients (*S* value) for pairwise analysis between different karyotype patterns were calculated using the formula $S = 2N_{AB}/(N_A + N_B)$], where N_A and N_B are the total number of bands in isolates *A* and *B*, respectively, and N_{AB} is the number of common bands (41).

Phylogenetic reconstruction. To estimate phylogenies, *URA5* sequences were aligned for analysis in Fig. 3 (left panel) and 4 by using Clustal W (1) Nucleotide sequences were used after removing all alignment gaps. These trees were constructed using the neighbor-joining algorithm (p-distance method), and molecular evolutionary analyses were conducted using MEGA version 2.1 (35). For analysis in Fig. 3 (right panel), URA5 DNA sequences were aligned using T-COFFEE (44) and the ends were trimmed to remove sparse columns. Phylogenetic reconstruction compared the sequences with introns in place and introns removed (29). Three different methods were used: one PAUP* neighbor-joining (reconstruction with p-distance (bootstrap, 10,000 replicates), one PAUP* parsimony bootstrap reconstruction treating gap characters as a fifth base (bootstrap, 1,000 replicates) (54), and two identical MrBayes reconstructions with the Hasegawa-Kishino-Yano model (31), 4 rate categories for gamma distribution (10 million generations, burn in conservatively set at 1 million generations; sample every 100 generations).

RESULTS

Phenotypic characterization of Indian isolates. We performed molecular typing on 57 clinical isolates from India that were cultured from a total of 39 patients (Tables 1 and 2. Eighteen isolates represented sequential isolates (defined as isolates recovered from a patient any time after the primary isolate was grown) on 14 of the 39 patients (Table 3). Of these patients, 46% were infected with human immunodeficiency virus. The remaining 54% had negative human immunodeficiency virus tests and had no known immune defects. Interestingly, all patients except one were male. The majority ($n = 47$) of isolates were grown from cerebral spinal fluid (CSF) specimens. Nine isolates were grown from other body fluids including blood ($n = 2$), urine ($n = 3$), sputum ($n = 3$), and wound aspirate ($n = 1$).

By M13 minisatellite RAPD typing, 5 isolates belonged to group VGII (*C. neoformans* var. *gattii*, serotypes B and C), 1

TABLE 2. Characteristics of *C. neoformans* (serotype B) strains from India

Strain	Age (yr)	Source of sample	Clinical presentation	HIV status	Pattern determined by:		MIC FLU[f]	Mating type
					CHEF	RFLP		
I104.97	25	CSF	CME[a]	Negative	XVII	R	8	α
I106.97	30	CSF	CME	Negative	XVII	R	8	α
I107.97	17	Aspirate	NA[b]	Negative	XVII	R	8	α
I49	68	Sputum	FUO[c]	Negative	XVI	P	16	**a**
I67 (NP-1)	34	CSF	AS[d], HA[e]	Negative	SM-XVIII	SM-S	16	α
					MC-XVIII	MC-S	16	α

[a] Chronic meningoencephalitis.
[b] NA, not determined.
[c] FUO, fever of unknown origin.
[d] AS, altered sensorium.
[e] HA, headache.
[f] MIC of fluconazole.

isolate (I114) belonged to group VNIV (*C. neoformans* var. *neoformans*, serotype D), and the remaining 51 isolates belonged to group VNI (*C. neoformans* var. *grubii*, serotype A). This identification was confirmed by serotyping (rabbit sera) with a commercial kit (Iatron, Japan). One isolate, I19-MC, was untypable by this method. As expected, 90% of the Indian serotype A and B isolates exhibited a Matα mating type. Of the 52 serotype A and D isolates, 50 exhibited a smooth (SM) colony morphology and 2 isolates exhibited a mucoid (MC) colony morphology. All serotype B isolates exhibited an MC colony morphology. Two isolates (I19 and I67) exhibited both SM- and MC-type colonies. The polysaccharide capsule of all isolates was measured in an India ink suspension under the microscope, and the sizes ranged from 0.6 μm to 3.7 μm. The capsule size of CSF-derived isolates was comparable to the capsule size of isolates derived from other locations (2.1 μm ± 0.4 versus 2.8 μm ± 0.5l; $P = 0.2$). In addition, no significant decrease in capsule size was documented for sequential isolates from patients where pre- and posttreatment isolates were available ($n = 14$) (1.7 μm ± 0.6 versus 1.7 μm ± 0.8; $P = 0.8$).

Karyotype analysis. The molecular epidemiology of *C. neoformans strains* from India was investigated by analyzing their karyotypes by CHEF. Fifty-five *C. neoformans* isolates from India revealed 18 distinguishable karyotype patterns (15 patterns for *C. neoformans* var. *grubii* and 3 patterns for *C. neoformans* var. *gattii*). In two isolates (I88 and I90, both derived from the same patient), sufficient cell lysis could not be achieved to make chromosomal DNA suitable for CHEF. The number of chromosomes ranged from 9 to 13 for *C. neoformans* var. *grubii* and 10 for *C. neoformans* var. *gattii*. Representative karyotype patterns are shown in Fig. 1. A large percentage (46%) of *C. neoformans* var. *grubii* isolates exhibited karyotype pattern III (34%) or karyotype pattern I (12%) (excluding sequential isolates). Eight karyotype patterns were associated with only one isolate, and the other patterns each were associated with either two or three isolates. The distribution of karyotype patterns was not different between isolates from immunocompromised patients or immunocompetent patients. The average ± standard deviation *S* value for 15 distinguishable karyotype patterns (from serotypes A and D) was 0.41 ± 0.1 for 105 comparisons and thus significantly ($P < 0.001$, by *t* test) higher than the previously published (24) average *S* value of 10 distinguishable karyotype patterns in New York City isolates (*S* values, 0.25 ± 0.06 in 44 comparisons). In summary, less diversity of electrophoretic karyotypes was observed among Indian isolates than among New York City isolates.

RFLP analysis. Indian isolates were also typed by RFLP. All contained the TCN-1 transposon. TCN-1 RFLP analysis revealed 19 different patterns (patterns A to S) among the 57 isolates. Each pattern exhibited 5 to 13 DNA hybridization bands for *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* isolates and 4 to 9 bands for *C. neoformans* var. *gattii*. Representative TCN-1 RFLP patterns are illustrated in Fig. 2. The hybridization patterns differed greatly from each other and could be easily distinguished visually. The most predominant RFLP pattern was pattern B, which included 47% of serotype A isolates (excluding sequential isolates). Many RFLP patterns ($n = 12$) included only one isolate.

The TCN-1 transposon was also found in the serotype A and D reference strains ($n = 7$) from other geographical areas. Although TCN-1 was found in all Indian serotype B strains, it could not be detected in the majority of *C. neoformans* var. *gattii* strains from other geographical areas. TCN-1 transposon was not detectable either by Southern blot hybridization with a TCN-1 probe or by PCR amplification with TCN-specific primers in genomic DNA of 18 of 20 *C. neoformans* var. *gattii* strains. This collection included 12 serotype B strains from

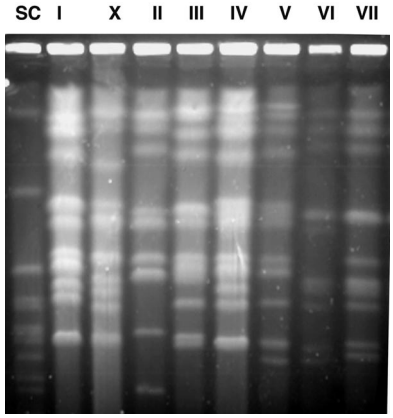

FIG. 1. Electrophoretic karyotype patterns of Indian *C. neoformans* strains. The majority of strains exhibit pattern I or III. (*SC* is a *Saccharomyces cerevisiae* chromosomal DNA molecular weight marker).

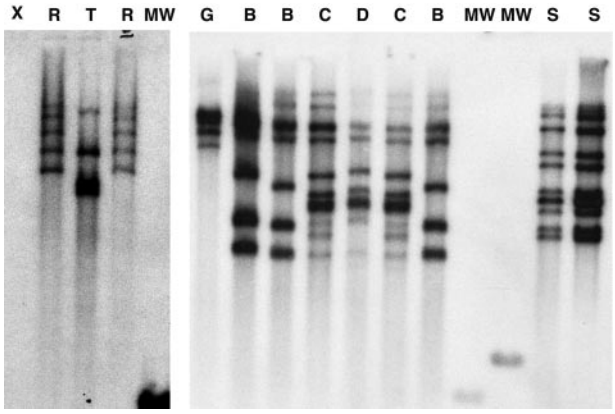

FIG. 2. RFLP patterns with the TCN-1 probe. Pattern B was the most common pattern for Indian strains. X denotes a *C. neoformans* var. *gattii* strain (AS 2557) that did not hybridize with the TCN probe. T is the only Australian *C. neoformans* var. *gattii* strain (AS1337) of 12 that hybridized with the TCN probe. (MW, 1-kb and 100-bp size markers.) The other letters denote RFLP patterns in *C. neoformans* strains.

Australia, 5 serotype B strains from Canada, and one serotype C strain from New York.

***URA5* sequence analysis.** Twenty-eight Indian strains (serotypes A and D) were compared with seven New York strains and five Brazilian strains (Fig. 3). The *URA5* sequences of serotype A strains and one serotype D strain were compared to those of ATCC 24064 (serotype A), B3501 (serotype D), five brazilian strains, and seven strains from New York City. The Indian serotype A strains fell into two major groups (Fig. 3, left panel). One group included the New York strains, and the other group included the Brazilian strains. Group 1 contained 28% (8/28) of Indian serotype A isolates (I108, I110, I121, I114, I116, I113, I57, and I14) and all the New York isolates (J15, J17, J24, J25, J19, J26, and J21) in addition to B3501. Neighbor-joining support for this group is 55%. MrBayes (Bayesian posterior probability [PP], 98%) (Fig. 3, right panel) and "bootstrapped parsimony" (bootstrap proportion, 51%) (data not shown) identify a similar group but keep the very dissimilar I57 and I14 as a separate group. Interestingly, Indian strains in group 1 that are for the majority serotype A (except I114) cluster with B3501, a serotype D strain. J21, a New York serotype A strain, also falls in this group. Relative to ATCC 24064, these strains had a mean number of base substitutions of 34.4 ± 18.8. Group 2 included 20 Indian strains, 5 Brazilian strains, and ATCC 24064. The Indian strains within that group had a mean number of base substitutions of 6.2 ± 3.9 relative to ATCC 24064. Most differences were found with the third position of the codon (silent substitutions). Because the introns are under a different selective pressure, we also analyzed the sequences after removal of introns and identified a similar grouping but excluded I57 and I14 for the first group (MRBayes PP, 76%; data not shown). Both RFLP pattern B (25% versus 50%, group 1 versus 2) and karyotype pattern III (12.5 versus 45%, group 1 versus 2) seemed to be more prevalent in group 2, although the correlation was not significant by the chi-square test.

With regard to *C. neoformans* var. *gattii*, 4 strains from India, 7 strains from Australia, and 6 strains from North America (including 5 from Canada) were compared to serotype B reference strain M93026. These strains clustered to some extent

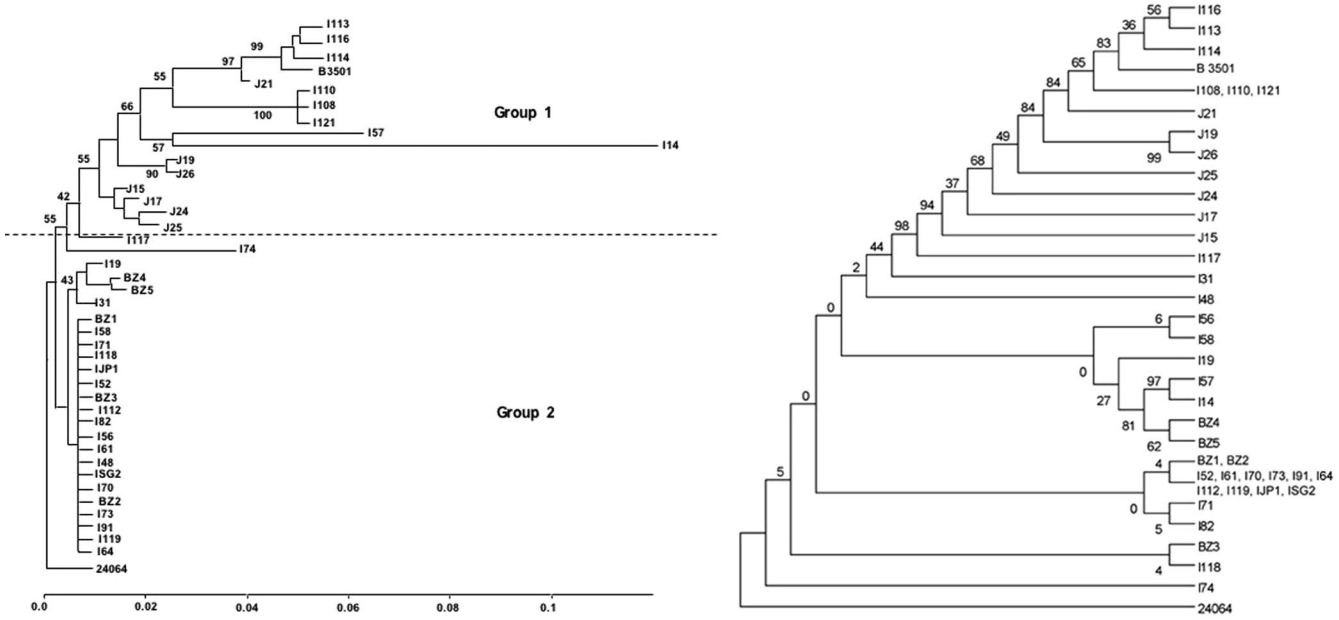

FIG. 3. (Left) A phylogenetic tree of 42 *URA5* DNA sequences from *C. neoformans* serotype A and serotype D isolates was constructed using the neighbor-joining algorithm (p-distance method) and rooted to ATCC strain 24064. Each number over a branch indicates the percentage of 1,000 bootstrap replicates that support the phylogenetic branch. The bootstrap values of <40% are not shown. Note that Indian strains fall into separate clusters: one is more closely related to the New York strains, whereas the other strains cluster with the Brazilian strains. (Right) Bayesian PP, 98% identifies a similar group but keeps I57 and I14 as a separate group.

TABLE 3. Characteristics of clinical serial *C. neoformans* isolates from India[a]

Patient no.	Strains	Days apart	Source of sample	Change in pattern of:		Change in MIC	
				CHEF	RFLP	FLU	AMB
1	I51, I52	d-1	CSF	No change	No change	16, 16	0.125
2	I55, I56, I60	d-3, d-45	CSF	III, III, IV	B, C, G	32, 32, 16	0.25
3	I57, I59	d-27	CSF	VII, VI	G, B	8	0.06, 0.25
4	I61, I63	d-7	CSF	No change	A, B	8, 16	0.25
5	I66, I73	d-131	CSF	IX, I	No change	32, 16	0.25, 0.5
6	I68, I74	d-99	CSF	XIII, II	No change	16, 32	0.125, 0.5
7	I82, I83	d-1	Blood, urine	No change	No change	16, 8	0.125, 0.06
8	I88, I90	d-14	CSF	ND	ND	2, 4	0.125
9	I91, I95, I96	d-6, d-15	CSF	No change	No change	16, 32, 16	0.125
10	I107, I110, I111, I112	d-8, d-8, d-13	CSF, urine	No change	C, D, C, C	8, 4, 4, 8	0.06, 0.125
11	I108, I109	d-2	CSF	No change	No change	8, 16	0.5
12	I114, I117	d-4	CSF	X, I	O, A	32	0.06, 0.125
13	I120, I121	d-5	CSF, blood	No change	No change	8, 16	0.125
14	I126, I129	d-18	CSF	No change	No change	8	0.125

[a] d, day; ND, not determined; FLU, fluconazole; AMB, amphotericin B.

according to their geographical origin (data not shown) and were found to exhibit a mean of 21.7 (range, 1 to 63) base substitutions in comparison with M93026. These clusters were maintained regardless of whether the strains carried the TCN-1 transposon or not. The percentage of identical sequence within the groups of isolates from a particular geographical region was 96% ± 1.9% for Indian strains, 94.2% ± 1.2% for Canadian strains, and 95.1% ± 5.4% for Australian strains.

Microevolution in *C. neoformans* isolates versus coinfection with distinct strains. Our strain collection also included a substantial number ($n = 18$) of sequential isolates from 14 patients, obtained during the initial diagnosis and persistence of cryptococcal infection (Table 3). For 5 of the 13 patients (for 1 patient, DNA could not be isolated), evidence for microevolution could be detected in the electrophoretic karyotypes of their isolates. In isolates from four patients, these karyotype changes were minor and involved chromosomal length polymorphisms in one to three chromosomes. Differences in RFLP patterns were also detected in 5 strains recovered from these 13 patients, 3 of which also exhibited changes in karyotype pattern. In the strains recovered from patient no. 2, karyotype changes were detected only in the third isolate (I60), whereas the RFLP patterns were different for all three sequential isolates. Two isolates recovered from patient 12 4 days apart yielded different karyotype and RFLP patterns (Fig. 4A). Strain I114 was

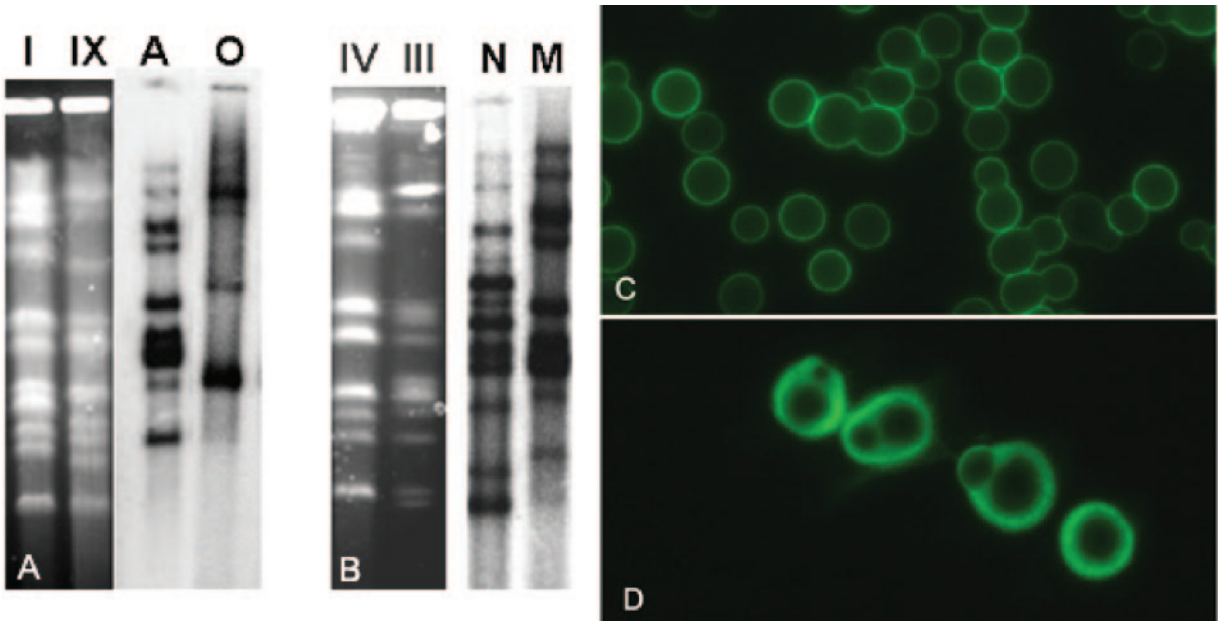

FIG. 4. Differences in karyotype and RFLP pattern are shown between I114 and I117 and between I19-SM and I19-MC. Panel A represents coinfection with a serotype A strain (I117) (karyotype I, RFLP O pattern) and a serotype D strain (I114) (karyotype IX, RFLP A pattern). Panel B represents karyotype and RFLP changes between an SM variant (karyotype IV, RFLP N pattern) and an MC variant of I19 (karyotype III, RFLP M pattern). The immunofluorescence staining of the polysaccharide capsule of the two colony variants revealed differences in binding by MAb 12A1 to the SM (C) and MC (D) polysaccharide capsules. *URA5* sequence analysis and sugar assimilation were identical.

typed as serotype D and strain I117 as serotype A. The *URA5* sequences of the two coinfecting strains differed by 34 nucleotides and clustered apart in the phylogenetic tree, consistent with their difference in varietal identity (Fig. 3). Assimilation of two sugars also differed between the isolates (data not shown). For patient 10, we also detected that one of the isolates (I112) recovered from urine at d-13 exhibited changes in RFLP pattern (C to D), whereas the karyotype pattern was unchanged. *URA5* sequence analysis revealed 33 nucleotide substitutions between strains I112 and I110, and accordingly, these were assigned to different clusters. These data also suggest that the strain in the urine was different from the strain recovered from the CSF. The MICs for amphotericin B and fluconazole were compared with sequential isolates. Sequential isolates from patients 3 and 6 exhibited a fourfold increase in MIC for amphotericin B. No change in MIC was observed for fluconazole. Interestingly, both sequential isolates with changed MICs also had karyotype pattern changes in the latter sequential isolate. The RFLP pattern was changed only in patient 3.

Variety in colony morphology (SM and MC) was observed with isolates I19 and I67 (sequential isolates were not available). With I19, the SM and MC colony phenotypes exhibited different RFLP and karyotype patterns (Fig. 4B). The *URA5* sequences of SM and MC were >99% identical (1 bp change). Furthermore, these isolates revealed identical sugar assimilation profiles and were both group VNI (serotype A) by M13 pattern; however, the MC isolate was not typable by the Iatron kit. Immunofluorescence staining of the polysaccharide capsule of the SM and MC variants of I19 revealed differences in binding by MAb 12A1 to the SM (Fig. 4C) and MC (Fig. 4D) polysaccharide capsules. We concluded that they were the same strain and had undergone significant changes in capsular phenotype, possibly as a result of phenotypic switching; alternatively, they could also be two distinct strains. In summary, we found evidence of coinfection with two strains for two patients and evidence for phenotypic microevolution, possibly as a result of phenotypic switching, for two patients.

DISCUSSION

In this study we investigated the molecular epidemiology of *C. neoformans* strains from patients in India. This study was undertaken because previous work suggested that patients in India exhibit a greater variety of clinical presentation of chronic cryptococcosis (2). We drew several important conclusions from our data. (i) Molecular data suggest that Indian strains are more homogenous than strains from other parts of the world. (ii) *URA5* DNA sequence comparison indicates that the majority of Indian strains fall into the same group as strains from Brazil, whereas a smaller fraction clustered with strains from New York City. (iii) *C. neoformans* var. *gattii* strains from India contain transposon TCN-1, whereas most *C. neoformans* var. *gattii* strains from other parts or the world do not. (iv) Chronic cryptococcosis can be complicated by microevolution or coinfection with two distinct *C. neoformans* strains.

The 57 Indian isolates included in this study were all clinical isolates and were predominately *C. neoformans* var. *grubii* isolates (serotype A) and a small number of *C. neoformans* var. *gattii* isolates (serotype B) and one *C. neoformans* var. *neoformans* strain (serotype D). This is consistent with reports that *C. neoformans* var. *grubii* is prevalent among immunosuppressed and healthy patients that are afflicted with cryptococcosis (43). It is noteworthy that we recovered only one *C. neoformans* var. *neoformans* (serotype D) isolate from India. *C. neoformans* var. *neoformans* is found primarily in temperate climates and appears to have lower thermotolerance, which may reduce its fitness in the tropics (39). Interestingly, more than half of the isolates were derived from patients that had no known impairment of their immune system. These findings are similar to a large survey of cases of cryptococcosis in Australia and New Zealand, which also found that 30% of the cases occurred in nonimmunosuppressed individuals and 26% occurred in immunocompromised patients without AIDS (17). The high proportion of nonimmunocompromised patients may allow comparisons between the molecular microevolution of *C. neoformans* strains over the next years in different patient populations and the effect of secondary antifungal prophylaxis in this process.

The Indian isolates were phenotypically similar to those isolated from other parts of the world. *C. neoformans* var. *grubii* isolates exhibited a smooth colony phenotype, whereas *C. neoformans* var. *gattii* isolates exhibited a mucoid colony phenotype. For two clinical strain isolates (a *C. neoformans* var. *gattii* isolate and a var. *grubii* isolate), more than one colony phenotype was observed in agar. Colony phenotype variability was also observed for other clinical specimens (25) and may be the result of phenotypic switching. This number may underestimate the prevalence of phenotypic variability or switching, since $<2 \times 10^4$ colonies per isolate were screened. Previous reports indicated that the polysaccharide capsule of *C. neoformans* in the brain is smaller than in the lung in a murine model of cryptococcosis (51), a finding that we could not confirm in this study. We also could not document a significant decrease in capsule size in sequential isolates pre- and posttreatment, although this had been shown in both in vitro (56) and in vivo (J. D. Nosanchuk, personal communication) studies.

Three DNA typing techniques were used to study the molecular epidemiology of the Indian isolates: electrophoretic karyotyping, TCN-1 RFLP, and *URA5* sequence analysis. Depending on the specific geographic area and the molecular typing technique, studies have shown different degrees of genetic heterogeneity among clinical and environmental isolates of *C. neoformans*, even within small geographic areas (6, 18). Electrophoretic karyotyping is a useful technique for distinguishing *C. neoformans* isolates, because there is great variation in the size and number of chromosomes among cryptococcal isolates (7, 47, 58). The number of electrophoretic karyotype patterns among the Indian isolates was lower than the number in previous studies of North American (34, 46) or European (20) isolates and similar to the variety reported for Brazilian strains (13, 22). For example, we found only 18 karyotype patterns among 55 isolates (derived from 39 patients), whereas Perfect et al. (46) found 41 patterns among 46 isolates and Dromer et al. (20) found 39 patterns among 40 isolates. In contrast, Franzot et al. (22) reported less heterogeneity, finding only 14 patterns in 51 isolates, and Calvo et al., in another Brazilian study, found 9 patterns in 25 isolates (13). Differences in CHEF technique do not account for this difference,

since a previous study from our laboratory in which the same methods and equipment were used found extensive karyotype diversity among isolates from one hospital in New York City (24).

In this study we demonstrate that a new RFLP probe, namely TCN-1, has high discriminatory power among clinical strains. The median average number of hybridization bands is slightly lower than that with the CNRE probe. The number of DNA hybridization bands ranged from 4 to 13, whereas CNRE patterns typically yield 14 to 20 DNA hybridization bands (53), which allows rapid and easy visual analysis of the TCN-1 RFLP patterns without computer programs. TCN-1 RFLP patterns exhibit excellent stability with initial and relapse isolates, and the probe can be easily generated by PCR amplification from cryptococcal DNA. Forty-three percent of the Indian serotype A isolates yielded one specific RFLP pattern with this TCN probe (pattern B); similar clustering is observed with the CNRE probe. This and other studies suggest that certain geographic areas exhibit less heterogeneity for *C. neoformans* var. *grubii* strains (22). These geographic differences in the molecular epidemiology of *C. neoformans* could result from natural selection by specific environmental and climatic conditions at individual geographic sites. Alternatively, the regions could have been colonized by different strains (founder effect) to begin with.

The relative homogeneity of the Indian isolates further supports the concept that *C. neoformans* strains have a predominantly clonal population structure (37). In this regard, the *C. neoformans* strains analyzed in this study appear to be different from those of the fungi *Coccidioides immitis* (12) and *Histoplasma capsulatum* (14), which have been reported to have a recombining population structure. A clonal population structure for *C. neoformans* is consistent with several biological characteristics of this fungus. *C. neoformans* exists as two heterothallic mating types, **a** and α, but sexual reproduction has been demonstrated only under appropriate laboratory conditions for a few strains. The overwhelming majority of clinical and environmental serotype A and serotype D isolates are of the α mating type, which decreases the chance of widespread sexual recombination in natural populations of *C. neoformans* var. *neoformans* and var. *grubii* (23, 36). In addition, Wickes et al. (57) demonstrated that infectious basidiospores could be generated by haploid fruiting without the occurrence of meiosis, which provides another mechanism for clonal reproduction in *C. neoformans* varieties *neoformans* and *grubii*.

Although *C. neoformans* var. *grubii* strains exist predominately as clonal isolates of the α mating type, there is evidence from molecular typing studies that certain pathogenic *C. neoformans* strains may be distributed worldwide. In this regard, parsimony analysis of the *URA5* sequences revealed a consensus tree where two-thirds of the Indian isolates fell into a separate group together with the Brazilian strains and separate from *C. neoformans* strains from New York City. Global dispersal of certain clones could occur as a result of wind transport and/or bird migrations. Birds like the common pigeon (*Columba livia*) were originally found in Europe and North Africa and have subsequently been introduced throughout the world by humans (32). Prevalence studies in the pellets of different avifauna in Madras detected *Cryptococcus neoformans* in 13.5% of pigeon droppings, 9.1% of fowl pellets, and 11.3% of crow droppings (27). Although the fungus does not colonize the gastrointestinal tract because of the higher body temperature, it can also be isolated from the beaks or the feet of pigeons (16). Both karyotyping and RFLP typing demonstrated the dominance of one pattern (III and B) in group 2. A statistically significant correlation between the molecular typing results could not be obtained. This may be due to a lack of power or may suggest that molecular typing techniques define independent molecular differences.

In contrast, *C. neoformans* var. *gattii* strains have a more restricted environmental niche. *C. neoformans* var. *gattii* is not associated with bird excreta but occurs in a varieties of trees including several *Eucalyptus* spp. (52). As with varieties *neoformans* and *grubii*, the population structure of variety *gattii* strains appears to be predominantly clonal, although both mating types exist and can generate new clades (23, 28). The majority *of C. neoformans* var. *gattii* strains are not fertile and cannot generate haploid spores by monokaryotic fruiting (57). Thus, *C. neoformans* var. *gattii* strains may not disperse as widely as those of variety *neoformans* and *grubii*, which may explain why the TCN element was not found in most strains, especially if sexual recombination is rare, as suggested by molecular typing studies with Australian strains (28). As determined by *URA5* sequence analysis, strains from the same geographical region tended to be more homologous.

We found a significant increase in MIC of amphotericin B in sequential isolates of 2 patients of 14 patients in whom sequential isolates were available. This number is slightly higher than previously reported (9, 11). Of note is that both strains also exhibited changes in karyotype patterns. Our data also show that human cryptococcosis can result from simultaneous infection with two strains, which may complicate treatment and contribute to failure. This observation appears to be the second well-documented case of human coinfection with two strains after the report by Haynes et al. (30). In addition, cryptococcosis in humans manifests as a chronic disease, and patients often are infected for weeks or months during which the fungus can undergo microevolution. During chronic infection, the host represents an environment that will promote selection of specific variants that result from microevolution. Microevolution is inherently difficult to identify and quantify (24). Nevertheless, careful analysis of sequential isolates in our study confirmed that *C. neoformans* strains undergo significant diversification in vivo as a result of microevolution. Significant karyotype changes have been previously described for switching *Candida albicans* and *C. neoformans* strains (26, 45). Interestingly, the recently published genome sequence of *C. neoformans* demonstrates that the genome is rich in transposons, many of which cluster at candidate centrometric regions (38). To what extent transposable elements could play a role in microevolution is unclear, and future studies are warranted to delineate the importance of this transposable element for the biology of this fungus.

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