Long-distance dispersal and recombination in environmental populations of *Cryptococcus neoformans* var. *grubii* from India

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The basidiomycete yeast *Cryptococcus neoformans* is a cause of significant morbidity and mortality in immunocompromised hosts throughout the world. The sporadic nature of the infection and the limited empirical evidence for direct human-to-human transmission have led to the belief that infections in humans are predominantly caused by the inhalation of basidiospores from environmental sources. Therefore, analysing the structure of environmental populations of *C. neoformans* can significantly increase our understanding of its ecology, evolution and epidemiology. Decaying wood is a rich source of organic and inorganic compounds and is known to be a suitable ecological niche for many micro-organisms, including *C. neoformans*. However, relatively little is known about the population structure of *C. neoformans* sampled from decaying wood. In this study, we analysed samples of *C. neoformans* var. *grubii* colonizing decaying wood in tree hollows of nine tree species in five geographical locations (Delhi, Bulandshahar, Hathras, Amritsar and Amrouli) in north-western India. Multilocus sequence typing was conducted using five gene fragments for each of 78 isolates. All isolates belonged to mating type α . Population-genetic analyses identified no evidence for significant differentiation among populations belonging to either different geographical areas or different host tree species. Interestingly, despite the lack of mating type α strains in our survey, we found unambiguous evidence for recombination in our population analyses. Our results are consistent with the hypothesis of long-distance dispersal and recombination in environmental populations of this species in India.

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INTRODUCTION

Cryptococcus neoformans var. *grubii* is the most common causative agent of cryptococcosis and can lead to significant morbidity and mortality in hosts with a defective T-cell-mediated immunity (Casadevall & Perfect, 1998). It has a global distribution and has been isolated from both human and environmental sources such as avian guano, soil, fruits, vegetables and decaying hollows of more than ten species of trees in different parts of the

world (Randhawa *et al.*, 2003; Reimão *et al.*, 2007). Current surveys suggest that the prevalence of cryptococcosis caused by strains of *C. neoformans* var. *grubii* varies among geographical regions, probably a reflection of their variable frequencies in different environments (Bennett *et al.*, 1977; Casadevall & Perfect, 1998; Tintelnor *et al.*, 2004). *C. neoformans* var. *grubii* represents strains of serotype A, one of the five serotypes (A, B, C, D and AD) defined based on their cell-surface antigenic properties within the pathogenic cryptococcal species complex.

Like those of other micro-organisms in nature, environmental conditions for *C. neoformans* may change and fluctuate significantly, both spatially and temporally, in nutrient levels, temperature, water availability, etc. Under conditions of low nutrient availability, low moisture and a temperature in the range of 15–33 °C, mating and sexual reproduction between strains of opposite mating types may occur, resulting in the production of basidiospores. These

Abbreviation: T-PTP, topology-dependent permutation tail probability.

The GenBank accession numbers for DNA sequences obtained here are: *CAP1*, EU312204–EU312281; *FTR1*, EU312282–EU312359; *ITS*, EU312360–EU312437; *LAC*, EU312438–EU312515; *mtLrRNA*, EU312516–EU312593.

A supplementary figure showing maximum-parsimony trees for the *LAC*, *CAP1* and *FTR1* markers is available with the online version of this paper.

sexual spores are smaller than vegetative cells and can disperse easily by wind or other means to other environmental niches, including human hosts.

The mating system of this heterothallic basidiomycete is controlled by one locus with two alternative alleles: *MATa* and *MAT α* (Kwon-Chung, 1975, 1976). Previous studies have shown that the majority of clinical and environmental isolates belong to the *MAT α* mating type (Kwon-Chung & Bennett, 1978; Yan *et al.*, 2002). Interestingly, a laboratory *MAT α* strain that belonged to serotype D (one of three serotypes, A, D and AD, in *C. neoformans*) has been shown to be more virulent than its congenic *MATa* strain in a murine model of cryptococcosis (Kwon-Chung *et al.*, 1992). Although the details and the relative frequencies of mating in natural environments are little known, in the laboratory, both opposite-sex mating (*MATa*–*MAT α*) and same-sex mating (*MAT α* –*MAT α*) have been demonstrated (Kwon-Chung, 1976; Keller *et al.*, 2003; Nielsen *et al.*, 2003; Lin *et al.*, 2005). A recent study indicated that the *MAT α* locus was among several genomic regions that contribute quantitatively to same-sex mating and haploid fruiting in *C. neoformans* (Lin *et al.*, 2006). Evidence for same-sex mating (*MAT α* –*MAT α*) in nature was recently reported between strains of serotypes A and D (Lin *et al.*, 2007). Same-sex mating has also been hypothesized to be responsible for generating the major genotype of a recent outbreak of cryptococcosis on Vancouver Island, British Columbia, Canada (Fraser *et al.*, 2005).

Sexual reproduction, between strains either of opposite sexes or the same sex, can potentially increase genetic variation in natural populations of organisms. The diversity of genotypes resulting from sexual reproduction can increase the efficiency of natural selection and may enhance the chances of successfully colonizing novel environments (Weismann, 1904; Goddard *et al.*, 2005). However, unlike those in the majority of plants and animals, where sexual reproduction can be easily detected, the detection of mating and sexual reproduction in micro-organisms requires the analysis of genotypic data from multiple strains in populations. Associations among alleles from within the same or different loci are used as indicators for sexual or asexual reproduction, typically through the analyses of multilocus linkage disequilibrium (Xu *et al.*, 1999; Xu, 2005b). In these tests, random associations (i.e. linkage equilibrium) among alleles at the population level are consistent with sexual reproduction while significant associations (linkage disequilibrium) are indicative of clonal reproduction. Other signatures of clonal reproduction include over- or under-representations of certain genotypes and genealogical congruence among genes from unlinked loci (e.g. Xu, 2005b).

Previous studies have employed a variety of molecular markers to demonstrate the modes of microbial reproduction in nature and to determine how evolutionary as well as ecological factors can influence the modes of reproduction and the structures of microbial populations (Maynard-Smith

et al., 1993; Avise, 1994; Xu, 2005b), including those of *C. neoformans*. For example, an MLST study of the population structure of *C. neoformans* var. *grubii* involving 102 representative strains from different parts of the world showed that the global population contained three genetically distinct subgroups (Litvintseva *et al.*, 2006). Ten of the 102 strains were *MATa* and they were all from Botswana. The remaining 92 were *MAT α* and were from different parts of the world, including Botswana. As expected, the Botswana population that contained strains of both mating types showed evidence of recombination and sexual reproduction (Litvintseva *et al.*, 2003, 2006). In contrast, the remaining geographical populations that contained only *MAT α* strains were largely clonal, with no clear evidence of recombination. Similar analyses of natural populations of a closely related species, *Cryptococcus gattii*, that contained exclusively or predominantly *MAT α* strains identified at least three distinct lineages in this species but no conclusive evidence for recombination (Kidd *et al.*, 2005). However, in another study of *C. gattii* populations, evidence for both clonality and recombination was obtained, with clonality found for samples belonging to one molecular type, VGI, and evidence of recombination found for samples of a different molecular type, VGII (Campbell *et al.*, 2005).

In this study, we analysed 78 strains of *C. neoformans* var. *grubii* isolated from nine tree species distributed in five geographical areas in north-western India. The mating types of these strains were determined based on direct PCR using mating-type-specific primers (Yan *et al.*, 2002) and all strains were found to have the *MAT α* mating type. A multiple-gene genealogical approach was used to analyse the population samples. DNA sequences were obtained for each strain from each of the five genetic loci distributed in different parts of the genome. Our population-genetic analyses identified no evidence of population subdivision based on either the host tree species or their geographical origins. Interestingly, while clonality and clonal dispersal among geographical regions were clearly evident in our analysed populations, there was also unambiguous evidence for recombination in this *MAT α* population of *C. neoformans* var. *grubii*.

METHODS

Samples of *C. neoformans*. The decaying wood inside the trunk hollows of nine species of trees was sampled for *Cryptococcus neoformans* (serotypes A and D) between 2003 and 2006. Strains were collected by taking swabs from the decayed wood and inoculating the swabs on modified niger seed agar (Randhawa *et al.*, 2005). The colonies were screened for morphological (microscopy) and physiological characteristics consistent with *C. neoformans* (VITEK 2 system, bioMérieux). Isolates were then serotyped using the Crypto-check slide agglutination test (Iatron). Details of the 78 strains obtained for this study are presented in Table 1. The strains analysed here are available upon request from Drs H. Randhawa and J. Xu.

DNA extraction and PCR amplification. A previously described technique (Xu *et al.*, 2000) was used to extract high-molecular-mass genomic DNA from all 78 strains. Fragments of four nuclear DNA

genes and one mitochondrial gene were chosen for PCR amplification and analyses. To avoid the potential bias in linkage disequilibrium due to physical linkage between markers, the five marker loci used in this study were located on separate chromosomes, as determined based on the completed JEC21 genome (Loftus *et al.*, 2005). The nuclear DNA markers used for amplification were: (1) *CAP1* (on chromosome 4) located within the mating locus and encoding a capsule-synthesis-associated protein; (2) *FTR1* (on chromosome 3), which encodes a high-affinity iron permease; (3) *LAC* (on chromosome 7), which encodes the laccase/diphenol oxidase; (4) the internal transcribed spacer (*ITS*) region, which comprises *ITS1*, 5.8S rRNA and *ITS2* (on chromosome 2); and (5) the mitochondrial large-subunit RNA (*mtLrRNA*) (Xu *et al.*, 2000). The JEC21 genome assembly from the Stanford Genome Technology Center (<http://www.tigr.org/tdb/e2k1/cna1/>) was used to identify the loci and design primers when required. Fragments of 731 bp (*CAP1*), 788 bp (*FTR1*), 486 bp (*LAC*), 468 bp (*ITS*) and 439 bp (*mtLrRNA*) were obtained by using the following primer pairs (5'→3'): *CAP1*, CGTTCGCGA-TAGAGAGAGGA (forward) and CCTTACCTTCACAGTCGCCC (reverse); *FTR1*, GTTCTCGGTACCACATCTTC (forward) and TCTC-AGGCTCGCCATCTTC (reverse); *LAC*, GGCGATACTATTATCGTA (forward) and TTCTGGAGTGGCTAGAGC (reverse); *ITS*, TCCG-TAGGTGAACCTGCGG (forward) and TCCTCCGCTTATTGAT-ATGC (reverse); *mtLrRNA*, CGTAAATACGATCCTGCATG (forward) and TTATCCCTAGCGTAACTTTTATC (reverse). Each PCR reaction comprised 2.1 µl (~1 ng) template DNA, 8 µl *GoTaq* (*Taq* DNA polymerase+MgCl₂ at a final concentration of 1.5 mM, supplied by Promega), and 0.2 µM of each primer to make up a total volume of 16 µl. The thermocycling profile included an initial denaturing temperature of 95 °C for 4 min followed by 40 cycles of 95 °C for 1 min, annealing temperature for 1 min, and 72 °C for 1 min. The final extension step was 72 °C for 7 min. Annealing temperatures for amplifying the five loci were: 59.2 °C for *CAP1*, 58.6 °C for *FTR1*, 50 °C for *LAC*, 50 °C for *ITS*, and 46 °C for *mtLrRNA*. The resulting PCR products were purified using the MicroCLEAN kit (Microzone) and sequenced at the MoBix Laboratory, McMaster University, using an ABI3100 automated DNA sequencer.

Mating type determination using molecular methods. The mating types of isolates were determined by PCR amplification using primer pairs designed from the sequences of the mating-type-specific *STE12* and *STE20* genes. These primers were serotype- and/or mating-type-specific. The specific primer sequences, amplification conditions, gel electrophoresis and data screening followed those described by Yan *et al.* (2002).

Phylogenetic analysis. Sequences from the 78 strains for each gene locus were aligned using CLUSTAL_X version 2 (Thompson *et al.*, 1997). The alignments were then imported into SeaView (Galtier *et al.*, 1996) and edited manually. Orthologues for the five genes in two model strains were retrieved for comparison from the completed genomes of serotype A (H99 strain, http://www.broad.mit.edu/annotation/genome/Cryptococcus_neoformans/Home.html) and serotype D (JEC21 strain, <http://www.tigr.org/>). Phylogenetic analysis was performed using PAUP*4.0b10 (Swofford, 1996). Maximum-parsimony trees were constructed for all the individual fragments by conducting heuristic searches based on 500 random sequence additions. Bootstrap analyses to assess the support for the clades were also conducted using 1000 replicate samples of phylogenetically informative characters. The serotype D strain JEC21 was used as an out-group for all the analysis. For the *LAC* gene fragment, where extensive sequence information is available and is used to delineate the molecular subtypes (VNI, VNII and VNB) within *C. neoformans* var. *grubii*, strains representative of each of the three types from Litvintseva *et al.* (2003) were analysed together with our data.

Clonality and recombination. Three complementary tests were conducted to examine evidence for clonality and recombination in the Indian population of *C. neoformans* var. *grubii*. In the first test, we examined evidence for genealogical incongruence among the five genes. This test was conducted using the partition homogeneity test (PHT) or incongruence length difference (ILD) test (Farris *et al.*, 1994) implemented in PAUP*4.0b10 (Swofford, 1996). The assumption made in this test is that the whole genome evolves as one single unit in strictly clonally reproducing organisms and as a result, genealogies of different gene fragments should be congruent in such organisms. In contrast, in the case of a recombining population structure, there would be incongruence among the different gene genealogies because different genes could have different histories. For this test, we used 100 replicates, each with 10 random sequence addition replicates using the NNI branch-swapping algorithm and saving a maximum of 1000 trees per replicate. This test was conducted for both the entire dataset and also the two largest individual populations, from Delhi and Amritsar, respectively. It has been shown that relying on this test alone for inferences of recombination could produce false-positive results when the consistency indices for individual gene trees are not high (Barker & Lutzoni, 2002). To avoid this potential problem, we also conducted the following two population genetic tests for evidence of recombination.

In the second test, we examined allelic associations among alleles from different loci using two common statistics in population genetics: the index of association (I_A) (Maynard-Smith *et al.*, 1993) and RbarD (Agapow & Burt, 2001) tests. I_A and RbarD were estimated using Multilocus, version 1.0b (Agapow & Burt, 2001). The I_A analyses the variance of the distances between all possible pairs of multilocus genotypes. The null hypothesis is that there is random association of alleles. Statistical significance was derived by comparing the observed dataset to 10 000 artificially recombined datasets. During the process of generating the artificially recombined datasets, we permitted random shuffling of alleles at each locus within a population but kept the proportions of alleles at each locus constant. If there were linkage disequilibrium due to a lack of recombination, the observed I_A should be significantly higher than the randomized recombining dataset. RbarD is a modified measure of I_A , but adjusted for the number of loci. The adjustment helps facilitate better comparison between populations and studies.

In the third test, we calculated the phylogenetic incompatibility by looking for the proportion of loci with all possible recombinant genotypes. This test looks for the evidence of recombination in a population by essentially looking for incongruence in different regions of the genome. The statistical significance of this test was inferred by comparing the number of incompatible pairs of loci in the observed dataset to those from a randomly shuffled dataset, using the program Multilocus, version 1.0b (Agapow & Burt, 2001).

Population structure. We assessed the genetic structure of the Indian samples of *C. neoformans* using three different analyses. In the first, we conducted the T-PTP (topology-dependent permutation tail probability) test (Faith, 1991) to determine whether host tree species or the geographical locations of strains had a significant influence on the topologies of phylogeny. For this test, reshuffled datasets were generated using Multilocus version 1.0b (Agapow & Burt, 2001) and the tree lengths were estimated using PAUP* version 4.0b10 (Swofford, 1996). If there was no significant structure identified with respect to either host tree species or geographical origins, the optimal tree score for the observed data without any constraint would be significantly shorter than for trees with constrained topologies. In the second test, population differentiation analysis was done by calculating θ (Weir, 1996), using Multilocus version 1.0b (Agapow & Burt, 2001). The null hypothesis of this test was no genetic differentiation between populations as defined by either host tree species or geographical

Table 1. *C. neoformans* var. *grubii* strains used in this study

Strain ID	Source tree/geographical origin	Year isolated	Multilocus genotype*				
INN3_279DDD_SC_DL_India†	<i>Syzygium cumini</i> /Delhi	2003	1	1	1	1	2
INN4_280U-DDD_SC_DL_India	<i>Syzygium cumini</i> /Delhi	2003	1	1	2	1	1
INN5_S284B_SC_DL_India	<i>Syzygium cumini</i> /Delhi	2003	1	2	2	1	1
INN6_320-1DDD'_SC_DL_India†	<i>Syzygium cumini</i> /Delhi	2004	1	3	1	1	1
INN8_320-2A_ii_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	4	2	1	1
INN9_320-3DDD'_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	1	2	1	1
INN11_320-4-DDD'_3_PL_DL_India†	<i>Polyalthia longifolia</i> /Delhi	2004	1	3	2	1	1
INN12_322AA_1_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	3	2	1	1
INN13_322AA_2_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	3	2	1	1
INN14_323A-AA_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	1	2	1	1
INN15_323A-DDD_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	1	2	1	1
INN16_323B-AA_2_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	3	1	1	1
INN17_323B-DDD'_1_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	3	2	1	1
INN18_323B-DDD'_2_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	3	2	1	1
INN19_325A_PL_DL_India†	<i>Polyalthia longifolia</i> /Delhi	2004	2	5	2	1	1
INN20_326B-DDD_1_PL_DL_India†	<i>Polyalthia longifolia</i> /Delhi	2004	1	1	2	1	1
INN26_373DDD'_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	1	2	1	1
INN27_374A/2-10_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	1	2	1	1
INN28_374A/2-100_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2004	1	3	2	1	1
INN29_540S-BB_1_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2005	1	1	2	1	1
INN30_540S-BB_2_PL_DL_India	<i>Polyalthia longifolia</i> /Delhi	2005	1	5	2	1	1
INN33_572S_ME_DL_India	<i>Mimusops elengi</i> /Delhi	2005	1	1	2	1	1
INN34_572S-C_ME_DL_India	<i>Mimusops elengi</i> /Delhi	2005	1	1	2	1	1
INN35_580A-d_ME_DL_India	<i>Mimusops elengi</i> /Delhi	2005	2	3	2	1	1
INN36_580A-S_ME_DL_India	<i>Mimusops elengi</i> /Delhi	2005	2	3	2	1	1
INN37_626S-C_AI_DL_India†	<i>Azadirachta indica</i> /Delhi	2005	1	6	2	1	1
INN38_627d-BB_AI_DL_India†	<i>Azadirachta indica</i> /Delhi	2005	2	1	2	1	1
INN39_628d-C_AI_DL_India†	<i>Azadirachta indica</i> /Delhi	2005	1	1	2	1	1
INN41_618A-d-C_MH_DL_India	<i>Manilkara hexandra</i> /Delhi	2005	1	1	2	1	1
INN43_618A-d-BB_MH_DL_India	<i>Manilkara hexandra</i> /Delhi	2005	1	1	2	1	1
INN44_618B-d-BB_MH_DL_India	<i>Manilkara hexandra</i> /Delhi	2005	2	1	2	1	1
INN46_611d-C_1_AN_DL_India	<i>Acacia nilotica</i> /Delhi	2005	1	1	2	1	1
INN48_692d-C_1_CF_DL_India	<i>Cassia fistula</i> /Delhi	2005	1	3	1	1	1
INN49_692d-C_2_CF_DL_India†	<i>Cassia fistula</i> /Delhi	2005	1	3	2	1	1
INN50_747S_2_CF_DL_India	<i>Cassia fistula</i> /Delhi	2006	1	1	2	1	1
INN51_748S_CF_DL_India†	<i>Cassia fistula</i> /Delhi	2006	1	2	2	1	1
INN52_748d_1_CF_DL_India	<i>Cassia fistula</i> /Delhi	2006	1	5	2	1	1
INN53_748d_2_CF_DL_India†	<i>Cassia fistula</i> /Delhi	2006	3	1	3	1	1
INN54_749S_1_CF_DL_India†	<i>Cassia fistula</i> /Delhi	2006	1	1	2	1	1
INN55_749S_2_CF_DL_India	<i>Cassia fistula</i> /Delhi	2006	1	1	2	1	1
INN56_750S_1_CF_DL_India	<i>Cassia fistula</i> /Delhi	2006	1	7	2	1	1
INN57_750S_2_CF_DL_India	<i>Cassia fistula</i> /Delhi	2006	1	1	2	1	1
INN58_751d_CF_DL_India	<i>Cassia fistula</i> /Delhi	2006	1	1	2	1	1
INN59_753d_1_CF_DL_India	<i>Cassia fistula</i> /Delhi	2006	1	1	2	1	1
INN60_753d_2_CF_DL_India†	<i>Cassia fistula</i> /Delhi	2006	1	1	2	1	1
INN61_757d_CF_DL_India	<i>Cassia fistula</i> /Delhi	2006	2	3	2	1	1
INN62_272DDD'_FR_DL_India†	<i>Ficus religiosa</i> /Delhi	2003	1	1	4	1	1
INN63_790S_1_A_SC_UP_India	<i>Syzygium cumini</i> /Bulandshahar	2006	1	1	2	1	1
INN64_806S_1_AI_UP_India†	<i>Azadirachta indica</i> /Bulandshahar	2006	1	1	5	1	1
INN65_809S_1_AI_UP_India†	<i>Azadirachta indica</i> /Bulandshahar	2006	2	1	2	1	1
INN66_713S_3_AI_UP_India	<i>Azadirachta indica</i> /Hathras	2006	1	1	1	1	1
INN67_717S_AI_UP_India	<i>Azadirachta indica</i> /Hathras	2006	1	1	2	1	1
INN68_425Sd-BB_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	1	1	1
INN69_425SS-C_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1
INN70_425SS-BB_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	2	1	2	1	1
INN71_428B-WC_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	2	1	2	1	1
INN72_428B-WC-BB_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1

Table 1. cont.

Strain ID	Source tree/geographical origin	Year isolated	Multilocus genotype*					
INN73_428B-WC-BBT_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN75_428B-WS-BB_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN76_430SS-C_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN77_430SS-BB_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN78_432WS-C_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	3	2	1	1	1
INN79_433Sd-C_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN80_436WS-C_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN81_437SS-BB_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	3	2	1	1	1
INN82_439WS-C_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	2	3	2	1	1	1
INN83_440SS-BB_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	2	3	2	1	1	1
INN84_441Sd-BB_1_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN85_441Sd-BB_2_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN86_441S-BB_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN87_441S-BB_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2004	1	1	2	1	1	1
INN88_449DDD_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2005	1	1	2	1	1	1
INN89_455DDD'_SC_PB_India	<i>Syzygium cumini</i> /Amritsar	2005	1	3	2	1	1	1
INN94_782d_1_MI_HR_India	<i>Mangifera indica</i> /Amrouli	2006	1	1	2	1	1	1
INN95_782d_2_MI_HR_India	<i>Mangifera indica</i> /Amrouli	2006	1	5	2	1	1	1
INN96_782d_3_MI_HR_India	<i>Mangifera indica</i> /Amrouli	2006	3	1	3	1	1	1
INN97_782S_1_MI_HR_India	<i>Mangifera indica</i> /Amrouli	2006	1	5	2	1	1	1
INN98_782S_2_MI_HR_India	<i>Mangifera indica</i> /Amrouli	2006	1	1	2	1	1	1

*Alleles for markers (from left to right) *CAP1*, *FTR1*, *LAC*, *ITS*, *MtLrRNA*.

†Indicates strains that have been tested for mating ability.

origins. Statistical significance for this test was derived by comparing the observed dataset to 10 000 randomized datasets. During randomization, any linkage disequilibria present in the observed data were maintained. The statistic θ ranges from 0 (for no population differentiation due to frequent gene flow) to 1 (total isolation of the populations from each other). In the third analysis, we examined the relationship between genetic distance and geographical distance between populations, using the Isolation by Distance Web Service (<http://ibdws.sdsu.edu/>) (Jensen *et al.*, 2005). A spatially limited gene flow would result in decreased genetic similarity between populations as their geographical distances increase, and this is commonly referred to as isolation by distance (IBD) (Wright, 1938). This test plots and correlates genetic distances and geographical distances between pairs of populations. The genetic distance measure used here is the standard Nei's genetic distance (D) between populations (Nei, 1972), established based on either host tree species or geographical origin.

Mating assays. The abilities to mate and reproduce sexually were examined for representative strains in this study. Briefly, 16 strains representing all 15 multilocus genotypes (MGs) identified in this study were chosen (see below; these strains are marked in Table 1). Each of the 16 strains was mated with three standard mating testers for *C. neoformans* independently. The three testers were JEC20 (*MATa*), JEC21 (*MAT α*) and H99 (*MAT α*). JEC20 and JEC21 belong to *C. neoformans* var. *neoformans* (serotype D) while H99 belongs to *C. neoformans* var. *grubii* (serotype A). To prepare for mating, strains were first grown on YEPD (yeast extract-peptone-dextrose) medium at room temperature for 3 days. Approximately equal numbers of cells from mating partners were then mixed on V8-Juice agar medium [5 % (v/v) V8 juice, 3 mM KH_2PO_4 and 4 % (w/v) agar at pH 7]. The plates were then incubated in the absence of light at room temperature ($\sim 22^\circ\text{C}$) for 21 days. For negative controls, we separately inoculated the three tester strains as well as the 16 representative strains from India onto V8-Juice agar medium and

incubated under the same condition as described above for mating mixtures. For positive controls, we mated JEC20 and H99. In addition, we constructed several pairs of same-sex matings between strains from India (all *MAT α*). Filamentation and basidiospore formation were recorded by light microscopy for each of the matings and controls.

RESULTS

Molecular variation

We were able to amplify all five DNA fragments for all the 78 isolates collected for this study. The results of PCR using the *STE12* and *STE20* gene primers showed that all isolates belonged to the α mating type. Our direct sequencing of PCR products identified no ambiguous nucleotide sites at any of the five genes for any of the 78 strains, consistent with haploidy. In total, we obtained 2912 nucleotides for each strain. The entire alignment had 11 parsimony-informative sites and 5 parsimony-uninformative sites. No polymorphic site was observed within the sequenced regions for the *ITS* and *mtLrRNA* gene fragments. The GenBank accession numbers for the 390 DNA sequences are: *CAP1*, EU312204–EU312281; *FTR1*, EU312282–EU312359; *ITS*, EU312360–EU312437; *LAC*, EU312438–EU312515; *mtLrRNA*, EU312516–EU312593.

We used both maximum-parsimony and maximum-likelihood analyses to construct the evolutionary relationships among strains for each of the three gene loci that

contained nucleotide polymorphisms, both individually (see supplementary Fig. S1, available with the online version of this paper) and in combination (Fig. 1). Both methods of analysis generated identical phylogenetic patterns. All the isolates clustered more closely with the *C. neoformans* var. *grubii* model strain H99 (serotype A) than with the *C. neoformans* var. *neoformans* model strain JEC21 (serotype D), consistent with our serological identification that all the strains analysed here belonged to serotype A. Aside from the *LAC* phylogeny, midpoint rooting was used to display the topologies of all the phylogenies to increase resolution among genotypes (Fig. S1 and Fig. 1). However, the topologies did not change when we rooted the trees using the orthologues from the serotype D strain JEC21 for either the *CAP1* or the *FTR1* gene tree. In each of the three polymorphic genetic loci, we saw no obvious evidence of strain clustering based on either geographical origin or host tree species (Fig. S1). Indeed, all major multilocus genotypes are shared among strains from multiple geographical regions or host tree species.

Among the 78 strains, the number of haplotypes for the individual gene fragments was as follows: four for *LAC*, three for *CAP1*, eight for *FTR1* (Fig. S1), and one each for *ITS* and *mtLrRNA* (Table 1). In total, the entire dataset yielded 16 multilocus genotypes among the 78 strains (Fig. 1). Almost all the genotypes identified here were represented in the largest subpopulation, from Delhi (Fig. 1). The second-largest subpopulation (Amritsar) had two haplotypes for each of *CAP1*, *FTR1* and *LAC*.

To compare the multilocus genotypes identified here with those reported previously, we compared our sequences with those in the GenBank using BLASTN. Our analyses identified that all our sequences had the closest matches to sequences of strains of *C. neoformans* var. *grubii*, consistent with our expectation. Of the three gene fragments analysed here, *LAC* has been the most widely used in previous studies. As a result, a significant number of allelic sequences exist for the *LAC* gene in GenBank and some of these sequences have been used to define several molecular types within *C. neoformans* (Litvintseva *et al.*, 2003, 2006). We therefore analysed our sequences with eight representative sequences from the three different molecular types to determine the likely molecular type(s) of our strains. The eight representative sequences of the *LAC* gene included five of VNI, two of VNB and one of VNII. Our analyses identified that all 78 strains showed the highest similarity to strains of the VNI molecular type (Fig. S1a).

Lack of genetic differentiation between populations

The θ value, a measure of population subdivision, showed no significant differentiation among geographical or host-tree-based populations and the observed value lies well within the distribution of the randomized datasets (only

results based on geographical origins are shown in Fig. 2). The amount of genetic differentiation observed between populations based on either geographical location (Table 2) or host tree species (Table 3) was also measured by Nei's *D* and we observed no significant difference between these populations. The lack of significant genetic differentiation is also supported by a lack of monophyletic pattern in the T-PTP tests based on either geographical origins or host tree species, with T-PTP values of 1.00 and 0.60 respectively. While we observed a positive correlation between geographical distance and pairwise population genetic distance, the correlation was statistically not significant (data not shown).

Evidence for both clonality and recombination

As described above, the evidence for clonality and recombination was examined using three tests. The first was the partition homogeneity test or incongruence length difference test. This test showed no statistically significant heterogeneity among the five markers used in this study. This test was conducted for the total sample of 78 strains as well as separately for each of the two largest geographical populations (Delhi and Amritsar). None of the samples showed any significant incongruence between genealogies from different markers [entire dataset ($n=78$), $P=0.27$; Delhi ($n=47$), $P=0.23$; Amritsar ($n=21$), $P=0.75$]. This result suggests that the overall population structure is consistent with significant clonality and clonal expansion. We also found other evidence for clonality based on the distribution of different genotypes between different geographical locations (Fig. 1). For example, two multilocus genotypes (MGs), MGIX and MGXI, were over-represented in the total sample with 41 and 10 isolates each, both from different host trees and geographical areas (Fig. 1). Furthermore, with the exception of MGIV and MGXVI (Fig. 1), all the other genotypes in our study were represented in the strains from Delhi. The MGXVI genotype was recovered from three different cities: Amritsar, Bulandshahar and Hathras. The lone genotype MGIV was from Bulandshahar. While genotypes MGI, MGII, MGIII, MGVI, MGVIII, MGX, MGIV and MGXV were exclusive to Delhi, the remaining genotypes were each sampled from more than one geographical location. This pattern of distribution and the sharing of genotypes between different geographical locations are consistent with long-distance dispersal and clonal expansion within and among the geographical populations of *C. neoformans* var. *grubii* from India.

The second test assessed the overall associations among alleles at different loci. Our population genetic analyses revealed that both I_A and R_{barD} values were low both for the total population sample as well as for the two largest subpopulations (Delhi and Amritsar) (Table 4). In fact, the null hypothesis of random recombination could not be rejected and the I_A values were well within the randomized recombining datasets (Fig. 3). We repeated these tests for

Fig. 1. Maximum-parsimony trees showing the relationships among 78 isolates of *Cryptococcus neoformans* var. *grubii* for the combined dataset using mid-point rooting (constructed using five markers: *CAP1*, *FTR1*, *LAC*, *ITS* and *mtLrRNA*). MPTs, most parsimonious trees; CI, consistency index; RI, retention index; HI, homoplasy index. Combined dataset (2912 bp aligned; 84 MPTs; tree length=20; CI=0.850, RI=0.953, HI=0.150). The combined dataset showed a phylogenetic pattern that did not distinguish populations based on either host trees or geographical locations. Genotype labels MG I to MG XVI represent the different multilocus haploid genotypes found in this study based on the five gene fragments. The midpoint-rooted tree is identical in topology to that based on an outgroup-rooted tree using sequences from the serotype D strain JEC21. The midpoint-rooted tree is shown to increase the proportional lengths of branches for better visualization.

samples specific to each host tree species and all failed to reject the null hypothesis of random recombination. More importantly, our third test, using phylogenetic incompatibility, also found clear evidence for recombination.

Specifically, among three polymorphic loci, *LAC*, *CAP1* and *FTR1*, we found all possible allelic combinations between pairs of loci for several alleles (raw data in Table 1 and examples shown in Table 5). Our results thus suggest

Fig. 2. Distribution of θ (a measure of genetic differentiation) among geographical populations of *C. neoformans* var. *grubii* from India. The θ value was well within the distribution of the permuted datasets that assumed no genetic differentiation ($P=0.366$).

that recombination is occurring within all populations analysed in this study.

Fertility of *C. neoformans* var. *grubii* strains from India

Among the 48 crosses that we set up between 16 representative strains and the three testers, only seven successfully produced hyphal filaments and basidiospores. All seven were with strain JEC20 (the *MATa* mating tester) and none with JEC21 or H99. The results are consistent with our molecular determination of mating types and suggest that at least some of the strains are sexually fertile. While the positive control cross JEC20 $\times$ H99 mated successfully, none of the negative controls worked. In addition, after more than 3 weeks of incubation, none of

the crosses between strains of the same sex, *MAT α* , produced any hyphal filaments.

DISCUSSION

C. neoformans var. *grubii* is among the most important fungal pathogens in humans. Understanding its population structure and ecology in natural environments could have significant medical implications. In this study, our population-genetic analyses showed evidence for extensive gene flow among the environmental populations of this organism in India. Furthermore, we identified evidence for both clonality and recombination in this sample, which contained exclusively the *MAT α* mating type. Our results are consistent with the hypothesis that *C. neoformans* might be able to complete its sexual life cycle on decaying wood of several tree species in India. A recent study by Xue *et al.* (2007) demonstrated that mating and sexual reproduction between strains of opposite mating types can occur for both *C. neoformans* and *C. gattii* on the model plant *Arabidopsis thaliana* and a natural tree host of *C. gattii*, the eucalyptus.

All of the host tree species investigated in this study are native to India. These tree species included *Syzygium cumini* (Java plum/jamun tree), *Polyalthia longifolia* (Indian mast tree), *Mimusops elengi* (bullet wood), *Azadirachta indica* (neem tree), *Manilkara hexandra* (margosa), *Acacia nilotica* (thorn mimosa), *Cassia fistula* (golden shower tree) and *Mangifera indica* (mango). In particular, one tree species, *P. longifolia*, is found around many homes throughout India. All the trees from which *C. neoformans* var. *grubii* was sampled were very close to human habitation. In addition to these tree species, *C. neoformans* var. *grubii* has also been recovered from the hollows of other tree species such as *Cassia grandis* (pink shower tree), *Senna multijuga* (November shower tree) and *Ficus microcarpa* (fig tree) (Lazera *et al.*, 1996). Our results demonstrated that the populations of *C. neoformans* var. *grubii* in different tree species are not genetically different and that there is frequent gene flow among different tree species. The spread of this micro-organism between different hosts could have been mediated by wind-aided dispersal of basidiospores or desiccated vegetative cells. The five cities from where the strains were isolated in this study

Table 2. Genetic differences among populations of *C. neoformans* var. *grubii* in India: Nei's genetic distance D between geographical populations

Geographical region	Delhi ($n=47$)	Bulandshahar ($n=3$)	Hathras ($n=2$)	Amritsar ($n=21$)	Amrouli ($n=5$)
Delhi 28° 36' 0" E 77° 19' 0" N	0				
Bulandshahar 28° 24' 0" E 77° 51' 0" N	0.053	0			
Hathras 27° 36' 0" E 78° 3' 0" N	0.076	0.058	0		
Amritsar 31° 37' 59" E 74° 51' 56" N	0.007	0.047	0.070	0	
Amrouli 28° 25' 59" E 77° 19' 0" N	0.038	0.084	0.095	0.048	0

Table 3. Genetic differences among populations of *C. neoformans* var. *grubii* in India: Nei's genetic distance *D* between populations from different host tree species

Host tree species	<i>Syzygium cumini</i> (<i>n</i> =26)	<i>Polyalthia longfolia</i> (<i>n</i> =14)	<i>Mimusops elengi</i> (<i>n</i> =4)	<i>Azadirachta indica</i> (<i>n</i> =7)	<i>Manilkara hexandra</i> (<i>n</i> =3)	<i>Cassia fistula</i> (<i>n</i> =14)	<i>Mangifera indica</i> (<i>n</i> =5)
<i>Syzygium cumini</i>	0						
<i>Polyalthia longfolia</i>	0.022	0					
<i>Mimusops elengi</i>	0.050	0.054	0				
<i>Azadirachta indica</i>	0.024	0.068	0.079	0			
<i>Manilkara hexandra</i>	0.024	0.082	0.065	0.031	0		
<i>Cassia fistula</i>	0.007	0.013	0.059	0.035	0.044	0	
<i>Mangifera indica</i>	0.046	0.053	0.118	0.064	0.067	0.028	0

are among the most densely populated regions in India. This is especially true of Delhi, where human population density and migration are both very high. The high rate of human migration could also contribute to the dispersal of *C. neoformans* var. *grubii* among different locations. In addition, tree-dwelling animals such as birds and squirrels can potentially aid in the spread and dispersal of this organism. At the global level, there is evidence for limited genetic differentiation and frequent gene flow among geographical populations of *C. neoformans* var. *grubii* (Xu *et al.*, 2000; Litvintseva *et al.*, 2003, 2006). Short- and long-distance dispersals have been reported previously for many fungal species, including saprobes, and plant and animal pathogens (Xu, 2005b).

The limited genetic variation found here among strains from the different hosts and geographical regions suggests that the Indian population of *C. neoformans* var. *grubii* might be of recent origin and/or that the dispersals were very frequent and recent. While the widespread over-representation of certain genotypes is consistent with clonality, the identification of phylogenetic incompatibility and low I_A values also suggested the presence of recombination in the Indian environmental population of *C. neoformans* var. *grubii*. These results are consistent with the structures of many microbial populations, characterized by unambiguous recombination, clonal dispersal and expansion of a few genotypes (e.g. Maynard-Smith *et al.*, 1993; Xu, 2005b).

The evidence for recombination in a $MAT\alpha$ population of *C. neoformans* var. *grubii* is surprising. Previous analyses of

samples of *C. neoformans* var. *grubii* have indicated that clonality was the dominant feature of reproduction in natural populations. This was somewhat expected because in most previous studies, the strains analysed from either human or environmental sources belonged to mating type α . There were two exceptions where evidence for recombination was detected in *C. neoformans* var. *grubii*. The first was an analysis of 14 hybrid strains of serotypes AD where both mating types *a* and α strains from serotypes A and D were reconstructed using gene genealogies. The reconstructed serotype A and D populations both showed evidence of recombination (Xu & Mitchell, 2003). The second example was from humans in Botswana, where both mating types were found and, not surprisingly, evidence for recombination as well (Litvintseva *et al.*, 2003).

Our current study is unique in that there was clear evidence for recombination in a sample where all the isolates belonged to one mating type, $MAT\alpha$. At present, the detailed mechanism for our observed recombination is not known. However, there are several possibilities. The first is that, unlike in most previous studies (e.g. Litvintseva *et al.*, 2003; Xu *et al.*, 2000), the samples analysed here were all from a geographically more restricted region, in north-western India. The close geographical proximity could have contributed to frequent mating and genetic mixing, resulting in recombinant genotypes. The second is that all strains analysed here are genetically very similar to each other. In fact, all 78 strains here were found to belong to the same molecular type, VNI. Strains with similar

Table 4. Multilocus linkage disequilibrium analyses for samples of *C. neoformans* var. *grubii* from India (subpopulations with sample sizes greater than 5 are also shown)

Population	I_A^* (<i>P</i> value)	RbarD (<i>P</i> value)	Phylogenetic incompatibility (<i>P</i> value)
Total (<i>n</i> =78)	0.1433 (0.138)	0.0360 (0.138)	0.5 (<0.0001)
Delhi (<i>n</i> =47)	0.1519 (0.102)	0.0385 (0.102)	0.7 (<0.0001)
Amritsar (<i>n</i> =21)	0.0454 (0.363)	0.0114 (0.363)	0.8 (0.108)

* I_A , index of association.

Fig. 3. Distribution of I_A values. The observed index of association (I_A) value was within the distribution of I_A obtained from 10 000 artificially recombined datasets ($P=0.1816$).

genotypes might have a greater opportunity of producing viable genetic recombinants capable of surviving in nature, as suggested previously by Campbell *et al.* (2005). The third is that the environmental niche, tree hollows, might be highly conducive to mating. A recent study indicated that both *C. neoformans* and *C. gattii* could complete their sexual reproductive life cycle in plants (Xue *et al.*, 2007). Most previous studies of environmental samples of *C. neoformans* var. *grubii* have focused on pigeon guano. Whether populations of *C. neoformans* var. *grubii* from different ecological niches (e.g. pigeon guano and soil) in

India show population structures similar to that from tree hollows remains to be examined.

The sexual recombination detected here could be achieved through one of two pathways. The first possibility is that recombination was due to same-sex mating between *MAT α* strains in natural populations of *C. neoformans* var. *grubii*. Same-sex mating has been observed in the laboratory (Lin *et al.*, 2005; Yan *et al.*, 2007) and in nature between strains of serotypes A and D (Lin *et al.*, 2007). The second hypothesis is that the recombination observed here was between strains of opposite mating types. Even though we were unable to isolate any *MAT α* strains from India, it is possible that *MAT α* strains are there but are very rare in nature in India and difficult to isolate. Our result that some of these isolates can mate with *MAT α* but not *MAT α* strains in the laboratory suggests that these *MAT α* strains might be present in the environment in India to help maintain the sexual fertility of *MAT α* strains. However, more extensive sampling would be required to distinguish between these two hypotheses. We would like to emphasize that the evidence for recombination was found not only for the whole sample but also for the subpopulations in Delhi and Amritsar. This finding suggests that many geographical regions in India (and potentially other places in other parts of the world) could be hotspots for sexual mating and the dispersal of basidiospores of this organism.

A combined sexual and asexual mode of reproduction is common for many microbial species, including many human pathogens (Xu, 2005b). It was recently shown that sexual reproduction could incur a cost in fitness (Xu, 2005a). It is also well known that sexual reproduction can produce a large number of recombinant genotypes that could speed up adaptation to novel environments (Weismann, 1904; Goddard *et al.*, 2005). In *C. neoformans*, while mating could occur between strains of the same or different sexes, it remains to be seen if the two alternative forms of mating have different adaptive significance. In addition, we know very little of the specific conditions in nature under which this organism undergoes same-sex mating as opposed to mating between opposite mating types. In the laboratory setting, low nitrogen, low moisture and ambient temperature are conducive to mating. The nutritional and other physico-chemical features of tree hollows from where *C. neoformans* were isolated are unknown at present. Ecological and physiological studies of natural environments conducive for *C. neoformans* mating will provide further insights into the mechanisms of evolution and population structuring of this important fungal pathogen.

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Table 5. Allelic combinations showing evidence of recombination in the environmental population of *C. neoformans* var. *grubii* from India

(a) All four possible allelic combinations are found between alleles 1 and 3 of locus *FTR1* and alleles 1 and 2 of locus *LAC*. (b) All six possible allelic combinations are found between alleles 1, 3, and 5 of locus *FTR1* and alleles 1 and 2 of locus *CAP1*. The number of strains for each genotype is shown in the table.

(a)	<i>LAC</i> (allele 1)	<i>LAC</i> (allele 2)
<i>FTR1</i> (allele 1)	4	42
<i>FTR1</i> (allele 3)	3	14
(b)	<i>CAP1</i> (allele 1)	<i>CAP1</i> (allele 2)
<i>FTR1</i> (allele 1)	43	4
<i>FTR1</i> (allele 3)	11	5
<i>FTR1</i> (allele 5)	4	1

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