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Isolates of *Cryptococcus neoformans* from Infected Animals Reveal Genetic Exchange in Unisexual, α Mating Type Populations▽†

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Sexual reproduction and genetic exchange are important for the evolution of fungal pathogens and for producing potentially infective spores. Studies to determine whether sex occurs in the pathogenic yeast *Cryptococcus neoformans* var. *grubii* have produced enigmatic results, however: basidiospores are the most likely infective propagules, and clinical isolates are fertile and genetically diverse, consistent with a sexual species, but almost all populations examined consist of a single mating type and have little evidence for genetic recombination. The choice of population is critical when looking for recombination, particularly when significant asexual propagation is likely and when latency may complicate assessing the origin of an isolate. We therefore selected isolates from infected animals living in the region of Sydney, Australia, with the assumption that the relatively short life spans and limited travels of the animal hosts would provide a very defined population. All isolates were mating type α and were of molecular genotype VNI or VNII. A lack of linkage disequilibrium among loci suggested that genetic exchange occurred within both genotype groups. Four diploid VNII isolates that produced filaments and basidium-like structures when cultured in proximity to an a mating type strain were found. Recent studies suggest that compatible α-α unions can occur in *C. neoformans* var. *neoformans* populations and in populations of the sibling species *Cryptococcus gattii*. As a mating type strains of *C. neoformans* var. *grubii* have never been found in Australia, or in the VNII molecular type globally, the potential for α-α unions is evidence that α-α unisexual mating maintains sexual recombination and diversity in this pathogen and may produce infectious propagules.

Recent studies provide experimental evidence for the benefits of sexual recombination that had been predicted based on theoretical analyses. Sex increases the genetic variation of populations, and by preventing linkage among loci, sexual recombination accelerates the elimination of deleterious mutations and reduces competition between beneficial mutations, allowing populations to respond to selection pressures (17, 18, 35). Sex between compatible mating partners can be difficult to achieve, however, particularly for nonmotile microorganisms that cannot actively find a mate. Among the fungi, many species appear to be asexual, and the majority of sexual species supplement sexual reproduction with prolific asexual propagation, which might be expected to result in predominantly clonal population structures. Nonetheless, a number of studies indicate that sexual recombination occurs in most fungal populations, including those of some species in which a sexual form has never been observed (7, 27, 56, 58).

From a pathogenic perspective, the exchange and reassortment of genetic information means that recombining populations have the capacity to adapt and change more readily than clonal populations, which may include expanding into new environments and developing new host specificities or the capacity to withstand antimicrobial treatments or host responses. For example, genetic recombination between an Australian strain and an unknown strain of the pathogenic yeast *Cryptococcus gattii* has been suggested to be responsible for a novel, hypervirulent genotype that has emerged in the Pacific Northwest of North America, a region where this fungus was hitherto almost completely unknown (25). Recombinant genotypes of the wheat pathogen *Mycosphaerella graminicola* are more frequently isolated from resistant wheat cultivars than nonrecombinants, suggesting that sex helps crop pathogens to overcome host resistance (73). Sex, then, appears to be important for the evolution and maintenance of most fungal species, further evidence for this conclusion being the complex mating systems many fungi employ to ensure the best chance of finding a compatible mate (14, 38).

The enigmatic yeast pathogen *Cryptococcus neoformans* does not fit neatly into this conceptual framework. This organism is a basidiomycete with two mating types: **a** and α (33, 39). Around 50% of isolates obtained from the environment or from human or veterinary patients with cryptococcosis are fertile and readily produce recombinant basidiospores in laboratory matings (40). Spores are thought to be important for the dispersal of *C. neoformans* and propagation in new hosts, as they are up to 100 times more infectious than yeast cells. Encapsulated yeast cells are too large to reach lung alveoli and thereby initiate disease, yet if the capsule is lost or reduced, cells rapidly lose viability and are no longer infectious (61, 69).

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Naturally occurring hybrid genotypes produced by the fusion of the related varieties *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are found, indicating that cross-varietal sex can occur (2, 42). However, almost all *C. neoformans* isolates that have been found to date are of the α mating type only, with **a** cells extremely rare and restricted to a few limited geographic regions (44, 49, 64, 65). In addition, the majority of population studies find little evidence for recombination among *C. neoformans* genomes on local or global scales (3–5, 15, 23, 47, 50). How fertility is maintained and how infectious spores are produced in the absence of a compatible mating partner are therefore ongoing mysteries.

Considerable effort has been devoted to studying and understanding the epidemiology of *C. neoformans* since this species emerged in the 1980s as a major pathogen affecting individuals infected with human immunodeficiency virus. Capsular antigens divide *C. neoformans* populations into two major serotypes that have separate varietal statuses: serotype A corresponds to *C. neoformans* var. *grubii*, and serotype D corresponds to *C. neoformans* var. *neoformans*. The closely related *C. gattii* encompasses serotypes B and C (13). Hybrid isolates are almost all serotype AD and are usually diploid. *C. neoformans* var. *grubii* is by far the most common cause of cryptococcal infection worldwide, particularly in urban areas where high numbers of the yeast cells exist in weathered pigeon guano. Molecular typing further divides *C. neoformans* var. *grubii* populations into three distinct genotypes, designated VNI, VNII, and VNB (50, 52).

Results from seroepidemiological studies indicate that exposure to *C. neoformans* occurs early in life, around the time that young children become mobile (28). The most common route of infection is likely to be the inhalation of infectious basidiospores, and given that the sexual production of spores seemed unlikely, attention has been focused on whether these spores could be made by asexual processes. Wickes et al. (70) found that on special starvation medium, some isolates of the α mating type could be induced to produce filaments, basidia, and spores, a process that has been termed "haploid fruiting" and which was hypothesized to be mitotic and asexual (70). However, although they reported this to occur in all the pathogenic *Cryptococcus* species, no studies since have been able to induce fruiting in any isolates of *C. neoformans* var. *grubii*. Recently, haploid fruiting has been shown in laboratory crosses to be a form of sexual reproduction between α cells (45), and there is evidence of it occurring in some natural *C. neoformans* populations in which AD hybrids are common (46) and in *C. gattii* populations (25, 59). Recent findings suggest that it may also occur in environmental populations of *C. neoformans* var. *grubii* from India (32), but whether this is a restricted or a widespread phenomenon is not known.

Population genetic analyses can be used to find signatures of recombination in populations and hence indicate whether sex and, by inference, spore production have occurred (8). Such analyses have found evidence of recombination in certain populations of *C. neoformans* in which cells of the **a** mating type are found or in which serotype AD hybrids occur (49, 71). One recent study found evidence for genetic reassortment among environmental *C. neoformans* var. *grubii* populations of the VNI genotype (32). However, in most populations analyzed to date, there is strong linkage disequilibrium among alleles at genetic loci, indicating that these populations are largely asexual. The choice of population is critical in recombination analyses, as isolates in the sample must be obtained from a space that is sufficiently wide to prevent the inclusion of many clonally derived organisms but narrow enough that the inhabitants have the chance to meet and mate. Finding such a suitable population of *C. neoformans* yeast may be particularly difficult, as variable and often extended latency and the propensity of humans for travel mean that clinical isolates obtained from infected individuals in one region may have very different geographic or temporal origins (8, 26) and environmental populations may include many clone mates derived from the asexual reproduction of yeast cells.

The aim of this study was to use a geographically and temporally defined population of isolates from companion animals to assess whether sexual recombination among isolates of *C. neoformans* var. *grubii* was occurring. Animals were specifically targeted, as they have shorter life spans than humans (and thus less time for latency) and generally do not travel far from their places of domicile. As the animals presented with symptomatic cryptococcosis, we presumed that this condition was due to the inhalation of infectious propagules and the establishment of disease in the upper or lower respiratory tract. This scenario permitted us to determine if these veterinary isolates had been generated strictly by asexual reproduction or by some form of sexual process associated with genetic recombination. We report here evidence for recombination among isolates of *C. neoformans* var. *grubii* VNI and VNII. No *C. neoformans* var. *grubii* isolates of the **a** mating type have ever been reported in Australia, the site of our study, and serotype D and the AD hybrids are extremely rare, so this population is representative of most *C. neoformans* var. *grubii* isolates worldwide. A number of α-α diploids were found that produced hyphal filaments and basidia when exposed to acidic pH and an **a** mating type pheromone. We conclude that recombination is widespread in *C. neoformans* var. *grubii* populations and that this recombination is likely to be mediated by α-α mating.

MATERIALS AND METHODS

Isolates. Thirty-one isolates of *C. neoformans* var. *grubii* were obtained from the University of Sydney Faculty of Veterinary Science Culture Collection. All isolates were from cats and dogs with cryptococcosis living in Sydney, Australia, and surrounding regions and were obtained from 1996 to 2002 (Table 1). Reference strains of *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* representing each molecular type were obtained from Westmead Hospital Culture Collection. Congenic serotype D strains JEC20 (MAT**a**) and JEC21 (MATα) (41) were used for mating assays. JEC21 (haploid) and XL146 (diploid) were used as controls to assess ploidy.

DNA extraction. Chromosomal DNA extraction was based on the method of Wen et al. (67) with the following modifications: approximately 0.75 g (wet weight) of cells grown on Sabouraud's dextrose agar (Oxoid Ltd., United Kingdom) was collected, the protoplasting solution was made with 10 mg of Glucanex (Novo Nordisk, Denmark) per ml of SCS buffer (20 mM sodium citrate, 1 M sorbitol), and all centrifugation steps were performed at 12,000 × *g*. The DNA pellet was resuspended in 100 μl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

Mating and molecular typing. PCR primers MfαU (TTCACTGCCATCTTC ACCACC), MFαL (TCTAGGCGATGACACAAAGGG), STE20$\mathbf{a}_{SF}$U (TCCG ATTGCTGCGATTTGCC), and STE20$\mathbf{a}_{SF}$L (GCGCCTGCACCATAATTC ACC) were used in a coamplification reaction to assess the mating type (29, 30). PCR amplifications were performed with 50-μl reaction mixtures containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 5% glycerol, 0.2 mM deoxynucleotide triphosphate (Bioline, United Kingdom), 0.5 μM concentrations of each primer, 1 μl of *Taq* DNA polymerase,

TABLE 1. Isolates included in the analysis

Isolate designation[a]	Year of isolation	Host, host description, and site(s) and/or symptom of disease	Molecular type
571-148	1996	Cat, 5 yrs old, male; nasal cavity	VNI
571-152	1996	Cat, 3 yrs old, female; nasal cavity	VNII
571-154	1996	Cat; mandibular lymph node	VNII
571-157	1996	Dog; brain and meninges	VNI
571-160	1997	Cat; 6 yrs old, male; nasopharynges	VNII
571-161	1997	Dog; nasal cavity and lymph node	VNI
571-162	1997	Dog; nasal cavity and lymph node	VNI
571-168	1997	Dog, 3 yrs old, female; primary intestinal lesion that spread to brain	VNI
571-174	1997	Dog, 18 mos old; central nervous system	VNI
571-175	1997	Cat, 5 yrs old, female; enlarged lymph node (site not stated)	VNI
571-176	1997	Dog, 2 yrs old, female; enlarged submandibular lymph node	VNII
571-179	1997	Dog, 3 yrs old, female; brain, meninges, and eye	VNI
571-180	1997	Cat, male; nasal cavity	VNI
571-183	1997	Dog, 2 yrs old, female; central nervous system signs	VNI
571-187	1998	Cat, male; nasal cavity	VNI
571-193	1998	Cat, 12 yrs old, male; site of bronchoalveloar lavage	VNI
571-198	1999	Cat; nasal cavity	VNI
571-202	1999	Cat, 8 yrs old, male; nasal cavity and middle ear	VNII
571-215	1999	Cat, 3 yrs old, male; nasal cavity	VNI
571-213	1999	Cat, 5 yrs old, female; nasal cavity	VNII
571-216	1999	Cat, 8 yrs old, male; nasal cavity	VNII
571-217	1999	Cat, 4 yrs old, female; nasal cavity	VNII
571-218	1999	Cat, 6 yrs old, female; nasal cavity	VNII
571-220	2000	Dog, 18 mos old, male; lower respiratory tract/lung	VNII
571-227	2000	Cat, 3 yrs old, female; nasal cavity	VNI
571-250	2000	Cat, 7 yrs old, female; nasal cavity	VNI
571-257	2001	Cat, 5 yrs old, female; ventral jaw mass (lymph node?)	VNII
571-258	2001	Cat, 14 yrs old, female; nasal cavity	VNII
571-259	2001	Cat, 5 yrs old, female; disseminated; lesions most obvious in subcutis around hock	VNI
571-260	2001	Cat; nasal cavity	VNI
571-255	2002	Cat, 7 yrs old, female; nasal cavity	VNI

[a] All isolates were mating type α.

and 1 μl of template DNA. Amplification conditions for PCR were 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension step at 72°C for 7 min. All amplifications were carried out in a PerkinElmer 2400 thermal cycler. A total of 10 μl of each amplification product was electrophoresed at 10 V/cm in 2% agarose gels. The gels were stained with ethidium bromide and visualized by UV transillumination.

Restriction fragment length polymorphism fingerprinting was based on the technique described by Meyer et al. with minor modifications (52). The amplification of the *URA5* gene was performed with 50-μl reaction mixtures containing 1× PCR buffer, 0.125 mM deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.2 μM concentrations of each of the primers URA5 and SJO1, 2.5 U of *Taq* DNA polymerase (Roche), and 1 μl of template DNA. PCR amplification conditions were 94°C for 5 min followed by 35 cycles of 94°C for 45 s, 61°C for 1 min, and 72°C for 2 min, with a final extension step at 72°C for 7 min. PCR products were digested with Sau96I and HhaI for 3 h at 37°C and separated on 3% agarose gels by electrophoresis at 10 V/cm for 1.5 h. Standard strains of known molecular types were used as controls.

Mating assays. Mating was assessed using the serotype D tester strains JEC20 (**a**) and JEC21 (α). Strains were pregrown on yeast extract-peptone-dextrose (YPD) agar (Oxoid Ltd., United Kingdom) or Sabouraud's dextrose agar medium for 2 days. A small inoculum of cells was transferred using a sterile toothpick and patched onto V8 juice agar (made by mixing 5% [vol/vol] V8 juice and 3 mM KH_2PO_4, adjusted to pH 3 or 7 using HCl, with an equal volume of 12% [wt/vol] agar, giving a final medium of pH ~4 or 7.5). Each isolate was plated onto a single plate alone (to check for self-filamentation) and in a mixture with an approximately equal amount of either the **a** or the α tester strain. Plates were incubated at room temperature for 10 days in the dark and then assessed for filament and basidiospore formation by using a dissecting microscope (Leica M420; Leica Microsystems). Matings were scored according to the extents of the filaments, basidia, and basidiospores they produced, as follows: for filaments, 1 to 50, +; 51 to 200, ++; 201 to 500, +++; 501 to 1,000, ++++; and >1,000, +++++; for basidia, 1 to 10, +; 11 to 100, ++; 101 to 300, +++; 301 to 500, ++++; and >500, +++++; and for spores, 1 to 100, +; 101 to 500, ++; 501 to 1,000, +++; 1,001 to 5,000, ++++; and >5,000, +++++.

Staining and microscopy. Filaments, basidia, and basidiospores were examined via light microscopy using an Olympus BX50 microscope equipped with an Olympus DP70 digital camera and a 100× primary objective lens. A small strip of transparent Scotch tape was gently touched over the mating area of the inoculated V8 plate, transferred onto a microscope slide, and stained with lactophenol cotton blue for microscopic examination.

Propidium iodide staining used cells grown on YPD medium overnight and fixed overnight in 70% ethanol at 4°C. Cells were then washed with NS buffer (10 mM Tris-HCl, pH 7.6, 250 mM sucrose, 1 mM EDTA, pH 8.0, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM $ZnCl_2$) and stained with propidium iodide (10 mg/ml) in 0.2 ml of NS buffer containing RNase A (1 mg/ml) at 4°C for 4 to 16 h. Differential interference microscopy (DIC) images and fluorescent images were captured using a Zeiss Axioskop 2 Plus fluorescence microscope equipped with an AxioCam MRM digital camera (Carl Zeiss MicroImaging, Inc., NY).

FACS analysis. Ploidy was determined by fluorescence-activated cell sorting (FACS) analysis as described by Sia et al. (60) with minor modifications. Approximately 20 μl of cells was harvested following growth overnight in YPD medium, washed once in 1 ml of PBS buffer, and then fixed in 1 ml of 70% ethanol overnight at 4°C. Fixed cells were pelleted via centrifugation, washed once with 1 ml of NS buffer, and then stained with propidium iodide as described above. Stained cells (50 μl) were diluted in 2 ml of 50 mM Tris-Cl, pH 7.5 to 8.0, and sonicated for 1 min. Flow cytometry was performed with 10,000 cells, and results were analyzed on the FL1 channel using a Becton Dickinson FACScan apparatus.

AFLP fragment detection and analysis. Amplified fragment length polymorphism (AFLP) fingerprinting used the protocol outlined by Halliday and Carter (31) with the following modifications: for preselective amplification, 1 μl of straight ligation product was used, while for selective amplification, 5 μl of the preselective amplicons (diluted 1 in 10) was used. The following primer combinations were employed: for preselective amplification, primers EcoRI-T and MseI-C and primers EcoRI-G and MseI-G, and for selective amplification, primers EcoRI-TG-FAM and MseI-CA and primers EcoRI-GT-FAM and MseI-GT.

Selective amplification products were diluted 100-fold in sterile distilled water and analyzed on an ABI PRISM 3700 DNA analyzer located at the Sydney

University Prince Alfred Macromolecular Analysis Centre. Data collection, fragment sizing, and pattern analyses were done using GeneMapper software version 3.5 (Applied Biosystems) with reference to a 500 LIZ size standard. Fragments of 50 to 500 bp with electropherogram peaks of >400 fluorescent units were selected for the analysis. The reproducibility of the AFLP data was assessed by running two samples of each isolate through the entire protocol on two separate occasions, starting from the isolation of total genomic DNA.

MLST. Isolates 571-148, 571-152, 571-154, 571-160, 571-176, 571-216, and 571-218 (representing diploid and haploid VNII isolates and one randomly chosen VNI isolate) were selected for multilocus sequence typing (MLST) analysis with loci *CAP10*, *IGS1*, *MPD1*, *PLB1*, *SOD1*, and *TOP1* (50). Reactions were done in 100-μl volumes containing 1× PCR buffer, 5% glycerol, 0.2 mM deoxynucleotide triphosphate (Bioline), 0.25 μM concentrations of each primer, 1 μl of *Taq* DNA polymerase (Roche), and 1 μl of template DNA. Amplification conditions for PCR were 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 7 min. All amplifications were carried out in a PerkinElmer 2400 thermal cycler. PCR products were purified using a GFX PCR DNA and gel band purification kit (Amersham Biosciences) and were sequenced in both directions using an ABI 3700 or ABI 3730XL instrument at the Macrogen sequencing facility (Korea).

Data analysis. (i) AFLP and recombination analysis. Align2 software was used to compare duplicate AFLP profiles for each isolate. Only bands present in both profiles (which included >95% of all bands) were considered in the phylogenetic analyses. Binary matrices were produced using LecPCR (D. Thioulouse, S. Chessel, and S. Doledec, 2001). Align2 and LecPCR were obtained from the ADE-4 Web server (http://pbil.univ-lyon1.fr/ADE-4/microb/). For each polymorphic locus, there were two possible alleles, which were scored as 1 when the amplified fragment was present and 0 when the fragment was absent. A polymorphic locus was included in the analysis only if it was present or absent in 25 to 75% of the isolates. PAUP*-compatible files (62) were generated from the AFLP data by Multilocus version 1.2b (1). These files were used to produce unweighted-pair group method with arithmetic mean (UPGMA) and parsimony phylograms. Bootstrapping was undertaken using 1,000 replicates.

The index of association (I_A) and parsimony tree length (T_L) tests (1, 7, 51) were used to distinguish between recombining and clonal modes of reproduction and were implemented as described in reference 9. In both analyses, statistical support for clonality versus recombination was achieved by comparing values for the observed data set with those for 1,000 artificially recombining data sets, produced by randomly shuffling the alleles for each locus among members of the population. A difference between the observed and recombining data sets was taken as evidence for clonality, with a significance level set at P of <0.01, although it is acknowledged that this approach cannot rule out all recombination, which may occur in a predominantly asexual background. For a complete explanation of these methods, see reference 9.

(ii) MLST analysis. Sequences were automatically aligned using ClustalW (BioManager) and imported into GeneDoc (53), where they were manually edited. Phylogenetic reconstruction was performed using neighbor joining and maximum parsimony, implemented in PAUP 4.0b10 (62). Maximum-parsimony analyses were done under the heuristic search setting using the branch-swapping method; gaps in the sequence alignment were treated as an additional character state. By using tree bisection-reconnection as an algorithm, 70 trees were found and a strict consensus tree was produced. Bootstrapping was undertaken using 1,000 replicates for both neighbor-joining and parsimony data. Data were combined with sequences for 26 isolates of *C. neoformans* var. *grubii* VNI, VNII, and VNB published in reference 50.

(iii) Genetic differentiation. Differentiation between groups was assessed as θ, which is an estimate of Wright's Fst and is calculated as follows: $\theta = Q - q/1 - q$, where Q is the probability that two different genes in a population are the same allele and q is the probability that these two genes are different alleles. If two different populations have the same allele frequencies, Q equals q and θ equals 0; if the populations are fixed for different alleles, Q equals 1, q equals 0, and θ equals 1. Values were calculated from the AFLP data using Multilocus. The observed θ value was compared with values produced from 1,000 randomizations of the data, which simulates populations undergoing panmictic gene flow.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession numbers EU426884 to EU426925.

RESULTS

Recombination is evident in companion animal VNI and VNII populations. Molecular typing by URA5 restriction fragment length polymorphism found that 19 of the isolates (61%) were molecular type VNI and 12 (39%) were molecular type VNII. The infecting genotypes appeared to be slightly different for the two host species: approximately equal numbers of infections in cats were caused by each genotype (12 by VNI and 10 by VNII), and dogs had a higher proportion of VNI infections (7 VNI and 2 VNII), but this difference did not reach statistical significance ($P = 0.42$). A total of 143 polymorphic loci were generated from the two AFLP primer combinations, which were reduced to 48 when loci present or absent in <25% or >75% of isolates were excluded (see Table S1 in the supplemental material). Of these remaining loci, nine were polymorphic between but not within the two genotypes, nine loci were polymorphic in VNI but not VNII, and three loci were polymorphic in VNII but not VNI. Phylograms generated from the AFLP data based on UPGMA (a clustering method based on grouping isolates) and parsimony (an optimality method based on finding the tree that can accommodate sequence changes with the least number of steps) (57) were congruent, with each showing only a single supported branch separating the VNI and VNII groups. The UPGMA phylogram is shown in Fig. 1. There was no bootstrap support for any nodes separating isolates within the VNI and the VNII groups. No correlation between genotype and animal host species was evident on the phylogram. The placement of isolates on a map of the Sydney region indicated that the VNI genotype caused the majority of infections in rural areas and that all VNII infections were in urban regions (see Fig. S3 in the supplemental material). However, this finding may largely reflect the host species, as the VNI rural infections all occurred in dogs, which are probably more common than cats in rural areas. Genetic differentiation between VNI and VNII was calculated as θ of 0.643375 ($P < 0.001$), which is similar to the levels of genetic differentiation found among some clades of *Coccidioides immitis* and *Histoplasma capsulatum* that are considered to be phylogenetic species (6, 12, 22, 34).

I_A and T_L were calculated for the observed and artificially recombined data sets. I_A measures the degree to which physically unlinked markers appear to be genetically associated, as would be expected in clonal organisms that inherit their genome as an unrecombined unit. T_L attempts to fit the multilocus genotype data to a phylogenetic tree so that the least number of steps or character state changes are required (7). Clonally reproducing populations display well-resolved parsimonious phylogenetic trees with short lengths, whereas recombining populations require branch swapping to accommodate allele exchanges and result in longer, poorly resolved trees. When all 31 isolates and 48 loci were included in the analysis, the I_A and T_L values were significantly different from those of their associated recombining data sets, causing the null hypothesis of random recombination in this population to be rejected (Table 2; Fig. 2). The analysis was then restricted to the VNI and VNII subgroups only, which reduced the number of polymorphic loci (present or absent in 25 to 75% of the isolates) to 21 and 25, respectively. For the VNI population, I_A and T_L values fell within the range of values obtained for the artificially recombined data sets ($P = 0.1$ and 0.42, respectively), indicating recombination in this group. Likewise, I_A and T_L values for the VNII group could not be differentiated from the range of values obtained for the recombined data sets ($P = 0.66$ and 0.36, respectively), providing strong evidence that this

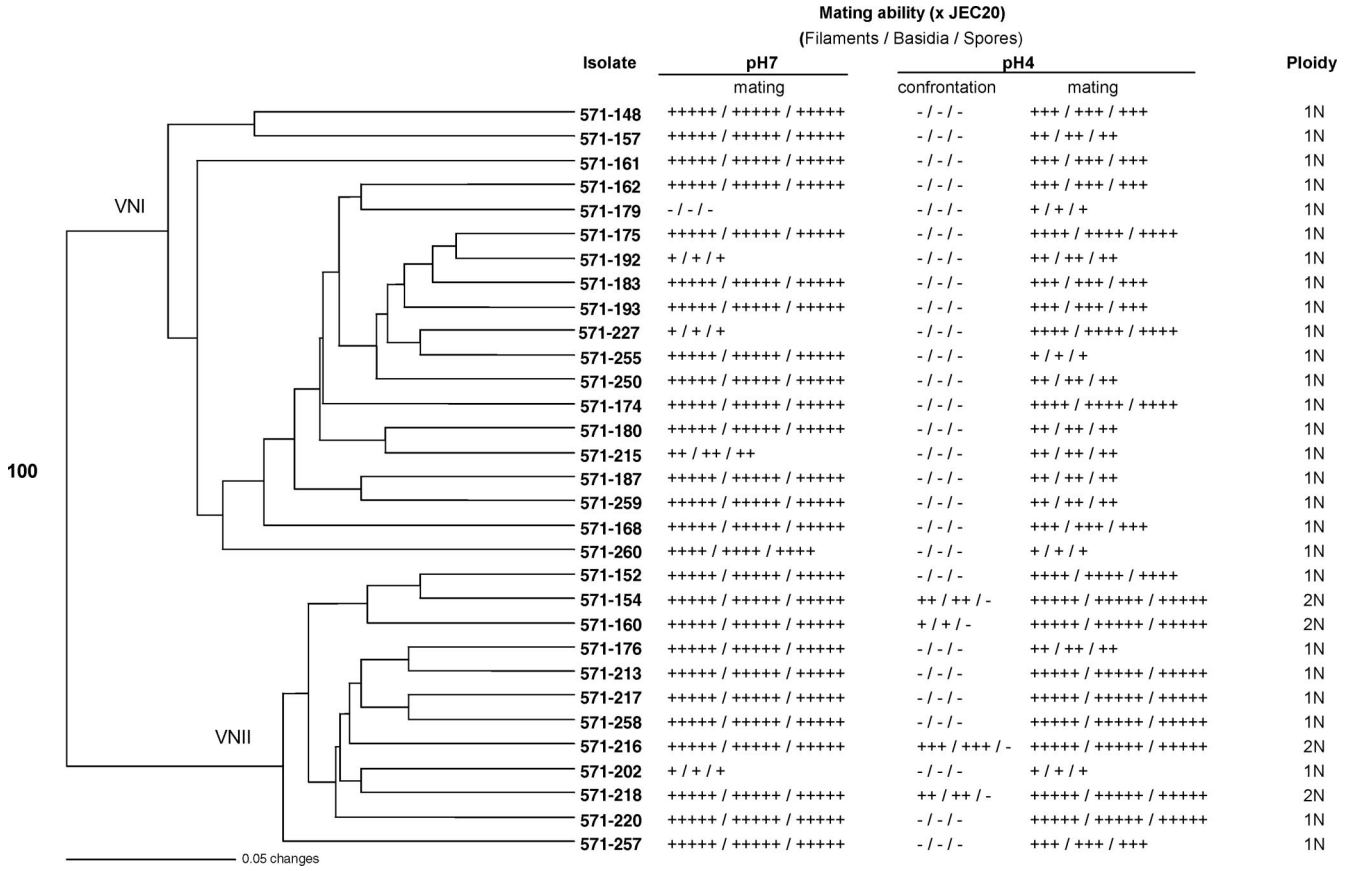

FIG. 1. UPGMA phylogram derived from AFLP data. The phylogram shows the relatedness of isolates included in the population, mating abilities, and ploidies (1N, haploid; 2N, diploid). To assess the mating ability, filaments, basidia, and basidiospores were scored from + to +++++ according to whether they were few to very profuse (see Materials and Methods for a complete description of scoring). − indicates the absence of the structure.

population has undergone genetic recombination (Table 2; Fig. 2). An analysis of character compatibility among all locus pairs indicated that most pairs had all possible combinations of alleles (i.e., 0-0, 0-1, 1-0, and 1-1) and that none had fewer than three of the four possible combinations (see Fig. S2 in the supplemental material), providing further evidence that the loci were independently reassorting.

Mating type analysis indicates that all isolates are mating type α. Coamplification using the MFα and STE20a_{SF} primers produced a 109-bp fragment from the α mating type control strain CBS5757 and a 219-bp fragment from the **a** mating type strain CBS6998. All of the *C. neoformans* var. *grubii* veterinary isolates produced a 109-bp α mating type-specific fragment, and none produced the **a** mating type-specific product. We therefore conclude that all isolates are of the α mating type. Mating plates were examined by microscopy for typical mating structures, including filaments, clamp connections, basidia, and basidiospores, and the results for each isolate are presented in Fig. 1. All of the isolates tested produced mating structures when mixed with JEC20, confirming them to be of the α mating type. Acidic medium generally suppressed the mating activities of isolates that mated robustly but enhanced the activities of some of the isolates that mated weakly. Acidic medium also stimulated the self-filamentation of four isolates (571-154, 571-160, 571-216, and 571-218), which produced filaments when grown in proximity to, but without physical contact with, the **a** mating type strain (Fig. 1 and 3A). Swollen

TABLE 2. Recombination analysis of *C. neoformans* var. *grubii* populations

Population (*n*)	No. of loci	I_A		T_L	
		Range[a]	Value[b] (*P*)	Range[a]	Value[b] (*P*)
All isolates (31)	48	−0.2–0.26	7.3905 (<0.001)	329–358	210 (0.001)
VNI (19)	21	−0.25–0.45	0.1511 **(0.1)**	83–98	91 **(0.42)**
VNII (12)	25	−0.4–0.65	−0.0746 **(0.66)**	65–80	72 **(0.36)**

[a] The range of values produced from 10,000 artificially recombining data sets.
[b] Value in the observed data set. *P* indicates whether this value is significantly different from values produced from recombining data sets; nonsignificant values indicate that the hypothesis of random recombination cannot be rejected and are shown in bold.

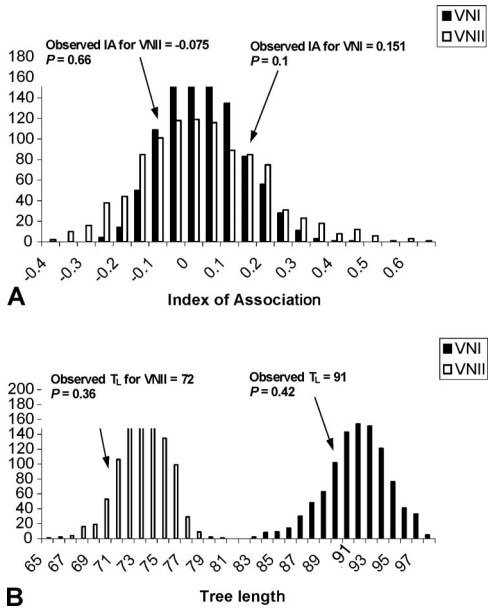

FIG. 2. Distributions of I_A and T_L values. Histograms show the ranges of I_A and T_L values obtained for 1,000 randomizations of the observed data sets for the VNI and VNII populations. Observed I_A and T_L values for each population lie within the randomized data sets.

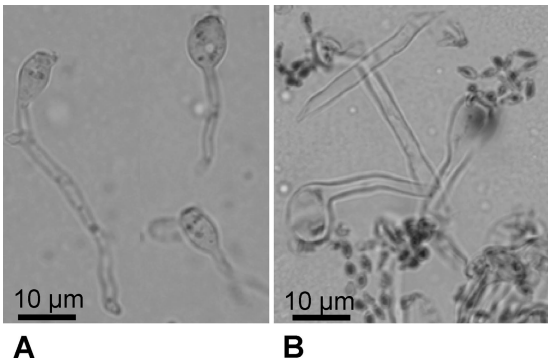

FIG. 4. Mating structures produced by diploids. Diploid isolates produced swollen hyphal tips resembling basidia but no spores when grown in confrontation with the **a** mating type strain. (A) Basidium-like structures produced by diploid isolate 571-154 grown in confrontation with JEC20. (B) The same isolate produced basidia and extensive spores following coculture and mating with JEC20.

hyphal tips resembling basidia were seen, but no spores or fused clamp connections were apparent (Fig. 4). Filaments could not be induced when strains were cultured on filamentation agar alone or in the presence of *C. neoformans* isolates of the α mating type or *C. gattii* isolates of the **a** mating type in a confrontation assay, in which confrontation was defined as growth in proximity to the opposite mating type without physical contact.

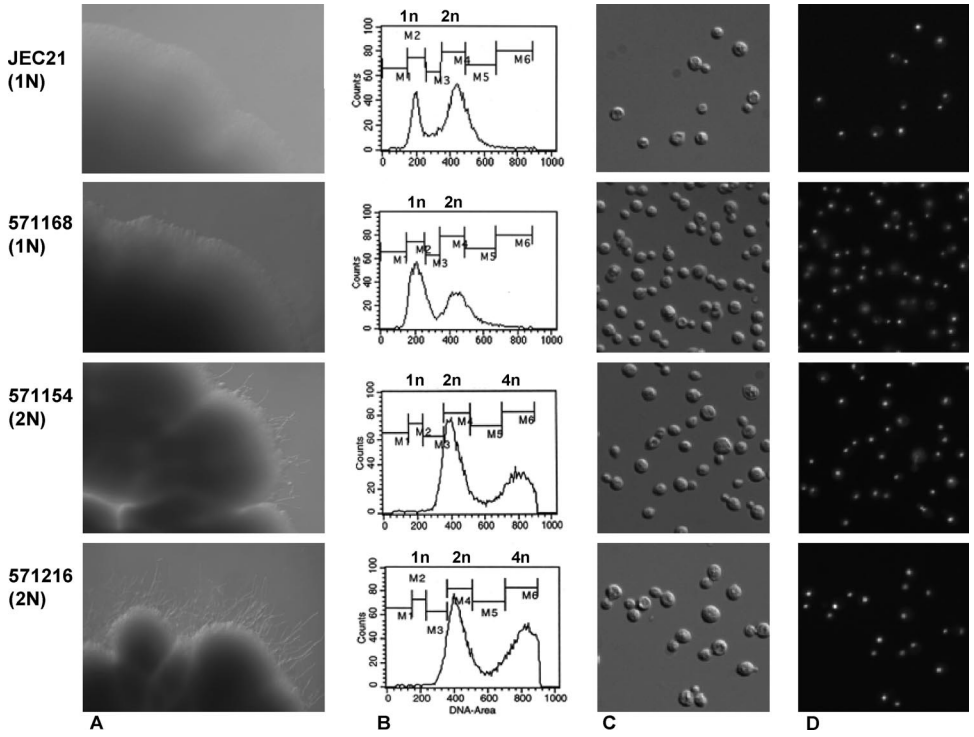

FIG. 3. Analysis of the diploid *C. neoformans* strains. Diploid (2N) isolates 571-154 and 571-216 are shown alongside haploid (1N) strains JEC21 (*C. neoformans* var. *neoformans*; α mating type) and 571-168 (*C. neoformans* var. *grubii* [this study]). (A) Filament formation in the confrontation assay with the **a** mating type *C. neoformans* serotype D strain JEC20. Diploids produced extensive filaments, which were not produced by JEC20 or by any of the haploid isolates. (B) FACS analysis indicated that self-filamenting strains have diploid genomes. 4N, quadriploid. (C) DIC microscopy images. Diploid cells had the morphology typical of *C. neoformans* encapsulated yeast cells but were slightly larger than haploids. (D) Fluorescence microscopy images of the same field shown in panel C. Diploid cells had a single nucleus.

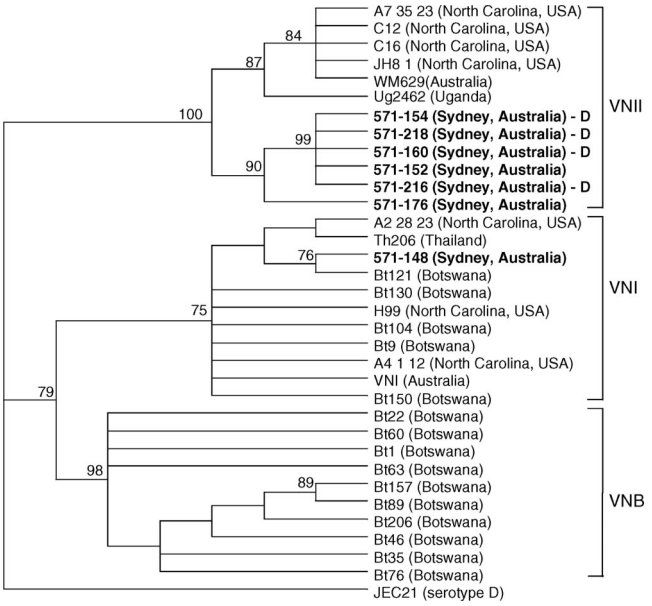

FIG. 5. Phylogenetic analysis of representative isolates compared with global VNI and VNII strains. The maximum-parsimony tree was derived from the sequences of six unlinked genetic loci. Isolates included in this study are shown in boldface; D indicates diploids. Australian VNII veterinary isolates formed a clade that was distinct from other VNII isolates.

Pairwise α-α crosses between pairs from a selection of the 31 veterinary isolates and between the self-filamenting strains and all other isolates were performed. No mating structures were observed in any of these crosses.

Self-filamenting VNII strains are diploid. FACS analysis found that the four strains that underwent self-filamentation on acidic medium had a diploid profile (Fig. 3B). The remaining 27 isolates were haploid. There was a significant association between ploidy status and the ability to self-filament among the VNII strains ($P < 0.001$). DIC and fluorescence microscopy found diploid cells to be slightly larger on average, with each cell containing a single nucleus (Fig. 3C and D). All diploids were from cats living in a restricted part of Sydney (see Fig. S3 in the supplemental material).

Sydney VNII isolates from infected animals are genetically distinct from global isolates of *C. neoformans* var. *grubii*. MLST analysis based on six unlinked loci was used to integrate representative isolates from the present study with a set of international strains described in reference 50 (Fig. 5). The VNII isolates formed a strongly supported group that was distinct from a second cluster that contained global VNII isolates. The latter included WM626, the standard VNII isolate which was obtained from a human immunodeficiency virus-infected patient in Sydney. Three of the four diploid VNII isolates (571-154, 571-160, and 571-218) were identical across the six sequenced loci; diploid isolate 571-216 differed from these by the deletion of a single adenosine residue (see Fig. S4 in the supplemental material). The three identical diploids were also identical to haploid isolate 571-152, apart from a single cytosine insertion, which was unique to this isolate. A number of single nucleotide polymorphisms and indels distinguished isolate 571-176 from the rest of the Australian veterinary VNII isolates. There was no evidence of heterozygosity in the MLST loci amplified from the diploid strains (see Fig. S4 in the supplemental material).

Australian VNI strain 571-148 grouped with strain bt121 from Botswana and was closely related to other VNI strains from the United States, Botswana, and Thailand. These were differentiated from the VNB cluster, which consists of Botswana isolates only (50).

DISCUSSION

Genetic reassortment occurs in natural populations of *C. neoformans* var. *grubii*. This study presents evidence of recombination among veterinary isolates of *C. neoformans* var. *grubii* belonging to molecular genotypes VNI and VNII. Clonality, as assessed by significant genetic linkage among loci, was rejected for both *C. neoformans* var. *grubii* genotypes, with the statistical tests unable to detect a difference between the structures of the observed populations and populations in which sexual recombination had been simulated by randomizing alleles at each locus among the isolates. Furthermore, pairwise combinations of loci found that all possible combinations of alleles occurred in the majority of cases, with a minimum of three of the four possible combinations found for each locus pair. Together, these results provide evidence that the populations have undergone genetic exchange via sexual recombination. There are two possible alternatives to this conclusion: first, homoplasy may have resulted in the independent loss or gain of markers among members of the population such that linkage disequilibrium was lost, and second, by focusing on subpopulations, we may have lost the statistical power required to accept the null hypothesis. Homoplasy appears to be highly unlikely in the present study, as this would be expected to remove all structure from the combined population, which was instead clearly divided into the different VN groups. To test whether the sizes of the two VN subpopulations had affected the outcome, we performed the analysis on simulated populations containing equal numbers of isolates from the VNI and VNII groups, and in all cases these populations were found to be clonal (data not shown). Published studies have found evidence for recombination in subpopulations containing fewer than 10 isolates (9, 51, 59). We therefore argue that there is evidence of significant genetic recombination among the veterinary isolates belonging to molecular genotypes VNI and VNII.

Most studies to date have found that, apart from a number of specialized populations, *C. neoformans* is globally clonal, a situation consistent with the overwhelming predominance of the α mating type (3–5, 15, 23, 47, 50). There are several features that distinguish this study and may have contributed to resolving recombining populations of *C. neoformans*. First, the analysis takes into account the genetically differentiated subgroups within the *C. neoformans* species complex, including different serotypes and molecular genotypes. In early studies, *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* were combined into a single analysis (3–5, 15, 23), and even when they were analyzed separately, only borderline evidence for recombination in populations of *C. neoformans* var. *grubii* could be detected, probably due to the genetic partition between the VN groups (63). Second, this study used high-resolution AFLP markers, which allow higher levels of polymorphism to be detected than those in many previous studies. The

low level of discrimination provided by the six MLST markers employed here would have likely indicated that the populations were clonal, a situation seen in our analyses of the closely related *C. gattii* (11). Third, the use of isolates that have actually caused clinical disease means that the populations in our sample are more likely to have been produced by sexually derived spores than populations from the environment, as samples from the environment may be biased toward yeast cells that are propagating asexually (31, 47, 50). Fourth, clinical samples derived from animals within a confined geographic range provide a very well defined population. In contrast, strains infecting humans may have been acquired far from the region where disease becomes clinically apparent. There is strong evidence that *C. neoformans* can parasitize and persist within macrophages (21) and that disease can result from the reactivation of a latent infection acquired many years before a clinical diagnosis is made (19, 26). Thus, studies incorporating predominantly human clinical isolates may be sampling from numerous, isolated populations (50). This effect may account for the differences seen between the VNII isolates from animals and WM626, which is also from Sydney but was obtained from an infected human who was born outside Australia and may therefore have acquired the infecting strain elsewhere. In a study similar to the present one, we found recombination within a veterinary population of *C. gattii* but we could detect recombination in human clinical isolate populations only by analyzing distinct, geographically restricted subpopulations (9). The recent study by Hiremath et al., which reported recombination in an environmental VNI population, also focused on a very defined geographic area (32). Finally, it is possible that our collection of isolates is unique and that it differs from other *C. neoformans* var. *grubii* populations. The veterinary isolates appear to have higher degrees of fertility than those reported in other studies of *C. neoformans* var. *grubii*, in which typically around 40 to 50% of isolates were fertile (32; J. Heitman, unpublished results). Preliminary MLST data indicate that the veterinary VNII isolates form a distinct group, and it is possible that this is a recombining subgroup within the larger, clonal VNII group. Further analyses of additional strains are required to confirm this finding and to determine if veterinary VNI isolates likewise differ from their global counterparts.

Diploidy and recombination suggest that α-α mating occurs in *C. neoformans* var. *grubii* populations. Recombination has been found previously in a single, unique population from Botswana in which both the **a** and α mating types occur. This population, known as VNB, is genetically distinct from both VNI and VNII, which were found in the same study to be clonal (50). Recombination has also been detected by analyzing genealogies derived from genes contributed by the *C. neoformans* var. *grubii* or *C. neoformans* var. *neoformans* parent in serotype AD hybrids (71). Hybrids can result from either Aα × D**a** or A**a** × Dα crosses, and the presence of **a**ADα isolates in this collection suggested that the **a** mating type might be more common in the environment than was previously thought. Recent evidence, however, has found representative **a**ADα strains from the United States, China, and Europe to belong to the VNB subgroup, suggesting that these strains originated from the same recombining population reported in Botswana (48). Both *C. neoformans* var. *neoformans* and AD hybrids are very rare in Australia (36), and it is not known if any **a**ADα strains exist. We therefore propose that mating is occurring among α mating type strains of *C. neoformans* var. *grubii* isolates that are endemic in Australia.

The *C. neoformans* var. *grubii* genotype VNII population had both significant evidence of genetic reassortment and a high percentage of diploid isolates. While a very small number of VNI **a** mating type isolates have been found (~3 in 3,000, or 0.1%), 100% of the VNII isolates examined to date have been of mating type α. Globally, VNII is less common than VNI (20), but it has been isolated from all of the inhabited continents and is a common cause of cryptococcosis (36). Litvintseva et al. (47) surveyed a large collection of environmental and clinical *C. neoformans* isolates and found that *C. neoformans* var. *grubii* isolates divided into two molecular subgroups, which by later analysis were found to correspond to VNI and VNII (48). Over 97% of all environmental *C. neoformans* var. *grubii* strains were VNI, but there were statistically equal numbers of clinical isolates of VNI and VNII. The authors felt it was unlikely that the environmental isolation procedure, which derived isolates from pigeon guano, had biased the isolation of VNI and speculated that either there was an alternative environmental reservoir for VNII or isolates of this genotype were more likely to cause infection (47). Our preliminary MLST analysis of a subset of VNII isolates indicated that these were restricted in diversity and were distinct from the global VNII isolates analyzed by Litvintseva et al. This finding may be evidence that recombination is occurring on a local scale only.

There are interesting parallels between *C. neoformans* VNII and *C. gattii* VGII, which is less common and more geographically restricted than *C. gattii* VGI (37), is largely fertile with a recombining population structure (9, 10), has a yet undetermined environmental reservoir but appears to cause a disproportionately high prevalence of disease in some regions (16), and in most regions appears to be exclusively of the α mating type (11).

Thirty percent of the VNII strains were α/α diploids. **a**/α diploids arise frequently from **a** × α crosses, forming ~1% of all viable basidiospores (60, 68); upon germination at 24°C, these basidiospores are self-fertile and readily form filaments and recombinant spores (60). The VNII α/α diploids found here formed filaments and basidium-like structures but no spores. Spore formation was seen only when the isolates were cultured at a low pH in confrontation with an **a** mating type *C. neoformans* strain, when the isolates were presumably responding to secreted **a** pheromone. No filaments were formed on filamentation agar, at low temperature, or in confrontation with the α mating strain. Filamentation helps cells forage for nutrients and is clearly advantageous in seeking a compatible mate. Pigeon guano is relatively acidic, a factor thought to contribute to the successful colonization by and propagation of *C. neoformans* (66), and may contain microenvironments that are sufficiently acidified to stimulate filamentation. However, as the morphological transformation of these diploids also requires proximity to compatible **a** mating type cells, its relevance in natural environments is not known.

Diploids arise following improper segregation at mitosis or meiosis or from cell-cell fusion and may contain two identical or two nonidentical haploid genomes. There was no evidence of heterozygosity at any of the six MLST loci or at five poly-

morphic microsatellite loci (data not shown). This finding may indicate that the nuclei are identical and have arisen through endoduplication, or it may be due to fusion between clonally related cells occurring in close proximity to one another. The overall low level of genetic diversity in the VNII population may also make heterozygosity difficult to detect. Lin et al. found natural αADα diploids among a collection of clinical *C. neoformans* isolates and suggested that the divergence of the *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* genomes may impair meiotic segregation and trap cells in the diploid state (46). It is also conceivable that the absence of the **a** mating type allele may prevent segregation in some genetic backgrounds, making diploids more common in α-α matings.

The evolution of α-α mating. Why would an organism with a functioning heterothallic bipolar mating system that would normally serve to promote outcrossing become unisexual and effectively homothallic? Few studies have examined functional differences between the two *C. neoformans* mating types, and although there is some evidence for a role of the α mating type in mammalian virulence (41, 54, 69), there is no evidence yet that it is associated with enhanced fitness in the environment. The *C. neoformans* mating type locus is particularly large and contains many genes (24, 43); conceivably, if any of these were to promote the fitness of one mating type over the other, this situation could lead to the rapid expansion of that type and the exclusion of the opposite mating type from the environmental niche that they share. Normally, the excessive expansion of one mating type only should be prevented because sexually recombining mixed-mating-type populations would maintain an evolutionary advantage (17, 18, 35). If the organism was able to maintain recombination among isolates of a single mating type, however, this advantage would be lost and the fitter, unisexual population could rapidly displace its bisexual counterparts. We currently do not know what governs α-α mating and whether this mating emerged before or after the α mating type became predominant. It would be interesting to compare the mating type loci and cell fitness and survival characteristics of α and **a** strains from the bisexual VNB population with those of α strains derived from recombining unisexual populations to test these hypotheses.

Although we now have evidence of genetic reassortment in *C. neoformans* var. *grubii* via α-α mating, it has not been possible to induce mating structures through α-α matings in the laboratory, nor have these ever been observed in the environment. **a**-α mating occurs between *C. neoformans* isolates on pigeon guano and in association with plants, and it would be interesting to test if these conditions can also stimulate α-α mating (55, 72). All isolates chosen for this study were from infected animals, and aerosolized spores produced by α-α mating may have been involved in the infectious process via inhalation. Alternatively, as animals live in closer association with soil and plant matter than humans, their infection by *C. neoformans* may differ and may involve the inhalation of soil or plant materials contaminated with the fungus. We have recently found evidence of recombination in environmental populations of *C. gattii*, including some that are exclusively of the α mating type, but as in the present study, we have not been able to induce mating structures in laboratory crosses between α isolates (59). Finding sex in an apparently unisexual population of *C. neoformans* and *C. gattii* may have parallels with the "cryptic sex" reported to occur in asexual fungi, in which genetic exchange occurs without an apparent induction of the perfect state (7, 27, 56, 58).

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