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DOI: 10.1534/genetics.109.113027

A Tetrad Analysis of the Basidiomycete Fungus *Cryptococcus neoformans*

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Manuscript received December 10, 2009
Accepted for publication February 9, 2010

ABSTRACT

Cryptococcus neoformans is a basidiomycete fungus that is found worldwide and causes disease in humans and animal species. The fungus grows asexually as a budding yeast. Under laboratory conditions it is capable of sexual reproduction between two mating types. After cell fusion a dikaryotic filament develops, at the tip of which a basidium gives rise to four chains of basidiospores. Because the chains each comprise 10–30 spores, rather than single spores, the analysis of individual meiotic events has not been attempted in *C. neoformans* in the style of tetrad analyses performed in other fungal species. Here, the basidiospores from >100 basidia were micromanipulated and the resultant >2500 progeny analyzed for three genetic markers to understand the sexual process in this fungus, leading to four observations: (i) Marker segregation provides genetic evidence for a single meiotic event within the basidium followed by multiple rounds of mitosis. (ii) Using each basidium as an unordered tetrad, the *ADE2* and *URA5* genes are linked to their centromeres, consistent with adjacent genomic regions rich in repetitive elements predicted to comprise Cryptococcus centromeres. (iii) Lack of germination of basidiospores is attributed to aneuploidy, rather than dormancy. (iv) Analysis of basidiospores derived from single chains demonstrates that each chain can contain different genotypes. This mechanism of sexual spore production would benefit the species with a high rate of dispersal and at the same time aid in simultaneous dissemination of both mating types to new locations in the environment.

CRYPTOCOCCUS neoformans is a fungus distributed worldwide and the cause of cryptococcosis disease in humans and animal species (Mitchell and Perfect 1995; Casadevall and Perfect 1998). The fungus grows as a budding yeast, which, as a morphological trait, hampered an understanding of its phylogenetic position within the fungal kingdom. In the mid-1970s a sexual cycle was characterized to demonstrate that the species belongs in the phylum Basidiomycota, because it generates a filamentous sexual state that results in spore production from a basidium structure (Kwon-Chung 1975). *C. neoformans* is divided into two serotypes and is closely related to *C. gattii* that also causes cryptococcosis. Unlike many other basidiomycetes that undergo meiosis to produce four spores, Cryptococcus and its relatives are distinguished by basidia with four long chains of attached spores. Cytological analysis of the nuclear behavior in mating reactions demonstrated that two nuclei are present in the filaments and are as such dikaryotic, while in the basidium either a larger diploid nucleus or multiple nuclei expected to be the products of meiosis are present (Kwon-Chung 1976). Mating occurs between cells of two mating types (*MAT***a** and *MAT*α) in a bipolar system (Kwon-Chung 1976), although a monokaryotic fruiting process of self–self mating can also occur under nutrient poor conditions (Wickes *et al.* 1996; Lin *et al.* 2005). Initial analysis of two genetic markers and subsequent Mendelian genetic analyses, leading to a full genetic map, demonstrated that meiosis occurs in the basidium (Kwon-Chung 1976; Marra *et al.* 2004). It is presumed that in each basidium there is a single meiotic event followed by rounds of mitosis to give rise to the chains of spores (Hull and Heitman 2002; McClelland *et al.* 2004; Lin 2009), although this process in the basidium has not been shown genetically.

In addition to its use as an experimental tool, mating in *C. neoformans* is potentially of medical relevance. First, it is hypothesized that the basidiospores are the infectious particles because they are sufficiently smaller in size than yeast cells, such that they would penetrate the lung alveoli to establish disease (Cohen *et al.* 1982), and recent studies have shown that spores are equally virulent as yeast cells (Giles *et al.* 2009; Velagapudi *et al.* 2009). Mating is supported by population genetic evidence of recombination (Litvintseva *et al.* 2003; Campbell *et al.* 2005; Campbell and Carter 2006) and the isolation of intervarietal (*e.g.*, serotype "AD") hybrids from the environment (Viviani *et al.* 2006). However, mating has not been described in the wild and most evidence suggests that if **a** × α mating does occur it would be rare, particularly for the more clinically common serotype A in which the *MAT***a** partner is found at present only in a

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small population in Botswana (Litvintseva *et al.* 2003). A second interest in mating is that there is a skew in the distribution of mating types in both wild and clinical isolates, with α being much more prevalent (Kwon-Chung and Bennett 1978) and an observation that in some genetic backgrounds the α strains are more virulent (Kwon-Chung *et al.* 1992; Nielsen *et al.* 2005). Addressing the basis for spores *vs.* yeast as the natural inoculum, how much mating occurs in the wild and skews in *MAT* allele frequency requires an understanding of the events that occur in the basidium.

Mating between **a** and α strains of *C. neoformans* can be induced readily under laboratory conditions and is an invaluable research tool using random spore analysis. Tetrad analysis, whereby the products of a single meiosis are analyzed, has never been reported in part due to the challenge in isolating and manipulating chains of spores rather than four or eight spores enclosed in the asci of model species like *Saccharomyces cerevisiae* or *Neurospora crassa*, or four spores on the basidia of *Coprinopsis cinerea*. Tetrad analysis provides a powerful tool to investigate different aspects of the meiotic process, including mapping of centromeres, detection of cytoplasmic inheritance and gene conversion, accurately measuring recombination frequencies, and was instrumental in formulating the current model for crossing over during recombination. Here, a tetrad analysis is applied for the first time to *C. neoformans*, demonstrating it is a feasible technique for this fungus and providing new insights into the reproductive biology of this organism.

MATERIALS AND METHODS

C. neoformans* strains:** *C. neoformans* var. *grubii* (serotype A) strains JF99 (*MATa** *ura5*) and M001 (*MAT*α *ade2*) were used as parents for crosses (Perfect *et al.* 1993; Nichols *et al.* 2004). The two strains are congenic, being derived from strains that had undergone 10 backcross events (Nielsen *et al.* 2003) and share identical DNA, with the exception of the alleles at the mating type locus and mutations within the *ADE2* and *URA5* genes, which result in the inability to grow in the absence of adenine or uracil, respectively. *C. neoformans* var. *neoformans* (serotype D) strains JEC21 (*MAT*α) and JEC20 (*MAT***a**) (Kwon-Chung *et al.* 1992) were used to test the mating type of progeny. A stable diploid (strain AI187) was isolated according to previous protocols (Sia *et al.* 2000) by mixing strains JF99 and M001 on mating medium and selection for cell fusion events on minimal yeast nitrogen base (YNB) medium at 37°.

Crosses, micromanipulation and progeny analysis: Crosses were performed or sporulation induced on Murashige-Skoog (MS) 4% bacto-agar (pH 5) media (Xue *et al.* 2007). Micromanipulation was conducted on a Nikon Labophot-2 microscope, equipped with a 25-μm optical fiber from Cora Styles Needles 'N Blocks (Hendersonville, NC). Plates with sporulating cultures were placed on the petri dish stage and the microfilament used to remove the spores of an individual basidium, or single spore chains, at once (Figure 1). Spores were then micromanipulated individually at 5-mm intervals on YPD agar medium supplemented with 20 mg/liter adenine. Plates were incubated at 30° for 3 days. Resultant colonies were velvet replicated to YNB medium supplemented with either uracil or adenine to score the auxotrophic markers and were crossed to strains JEC20 and JEC21 on 5% V8 juice agar (pH 7) supplemented with adenine and uracil to score mating type, as judged by basidiospore production viewed under a microscope. In situations of ambiguous phenotypes, progeny were single-colony purified and the three phenotypes reassessed.

Genomic DNA isolation, PCR and DNA sequencing: Genomic DNA was isolated from fungal cells either with a CTAB extraction buffer or NaCl-SDS buffer protocol (Pitkin *et al.* 1996). A fragment of *ADE2* was amplified by PCR with primers ALID0380 (ACTGGATCGACCTCATCG) and ALID0381 (CGATGACTGGCAAACTGG), and the full *URA5* gene was amplified with primers ALID0375 (TGCAAAGAGC GAAGTTGC) and ALID0376 (GATTGACAGCCAACAATC). Amplicons were sequenced and compared to the genome database for serotype A strain H99 at the Broad Institute. To assess the parental contribution of the mitochondrial genome, primers Da 20 (GACACTACACAAGATGCCTC) and Da 3 (GCAATAGCATATACCATCCCG) were used in PCR analysis that detects a size difference between the mitochondrial-encoded *COX1* gene of JF99 and M001 (Toffaletti *et al.* 2004). For amplification of the mating type alleles, primers ai144 (GAAAAGGTTACGACAGAGG) and ai145 (TGGGAA GAACTATCAGAGG) were used for the α allele (between the *FOA1* and *SXI1*α genes) and primers ai150 (ACGCAGTGT CAACTGGTC) and ai151 (CGCACCTTTTCTGTAGGG) used for the **a** allele (between the *RUM1* and *MYO2* genes).

RESULTS

Characterization and generation of *C. neoformans* strains: To examine the meiotic process in *C. neoformans*, strains were chosen that are congenic and contain markers that would enable genetic analysis of segregation. Two haploid strains of opposite mating type (*MAT*) bear mutations in genes resulting in auxotrophic phenotypes that can be scored easily. The *MAT* locus has two alleles, **a** and α, and comprises ~20 genes in a 100-kb region of the genome (Lengeler *et al.* 2002). Strain M001 (*ade2 MAT*α) is an adenine auxotroph that has been previously characterized to determine that it has a point mutation within the coding region of the phosphoribosylaminoimidazole carboxylase enzyme (Perfect *et al.* 1993; Sudarshan *et al.* 1999). The nature of the mutation within the *URA5* gene in strain JF99 (*ura5 MAT***a**), a uracil auxotroph, was unknown. The *URA5* gene was amplified and sequenced from this strain, and exhibits a single A- to G-bp substitution. This results in an aspartic acid to asparagine substitution at position 130, which is a residue conserved in orotate phosphoribosyltransferase enzymes in both fungi and bacteria. The mutant allele in strain JF99 alters a *Cla*I restriction enzyme site that can be used to score *URA5* alleles in diploid strains (*e.g.*, see Figure 3). Reversion rates back to wild type are low for both auxotrophs (Sudarshan *et al.* 1999 and my unpublished data). Analysis of the genome sequences of the wild-type serotype A strain H99 and serotype D strains JEC21 and B-3501 show that the *ADE2*, *URA5*, and *MAT* genetic loci are located on three separate chromosomes.

To generate a diploid serotype A strain, the M001 and JF99 strains were crossed on V8 juice agar and the cell mix plated onto minimal medium (YNB) and incubated at 37° to select for diploid yeast colonies (Sia *et al.* 2000). One strain, named AI187, was used in further studies.

The mitochondrial genome is different between strains JF99 and M001 and could potentially be used as a fourth genetic marker. From the crosses between JF99 and M001 (C1, C2, and C3 in Table 1), two progeny of each phenotype isolated were selected at random and their mitochondrial genotype assessed by PCR. All 20 progeny had the mitochondrial genome derived from the *MAT***a** parent (data not shown). The **a** mitochondrial genome is also present in the AI187 diploid strain (Figure 3). These findings confirm the previous observations about mitochondrial inheritance from the *MAT***a** parent in *C. neoformans* serotype D strains at the stage of cell fusion (Xu *et al.* 2000; Yan and Xu 2003) and excluded the mitochondrial genome as a useful independent marker in this analysis.

A single meiosis occurs in each basidium of *C. neoformans*: To examine the meiotic process during conventional mating, crosses were set up between the M001 and JF99 parents. Using a microscope for guidance, a micromanipulation needle was used to lift as much as possible of the chains produced from a single basidium (Figure 1). The attached basidiospores that are derived from a single basidium were then transferred to a YPD agar plate and separated from each other by micromanipulation, the basidiospores were allowed to germinate, and the three genetic markers were scored. Three basidia were analyzed (C1, C2, and C3 in Table 1; Figure 2). The rationale behind this genetic experiment is that if basidiospores are generated by a single meiosis occurring followed by rounds of mitosis, when analyzed for three genetic markers, each basidium should contain either two or four recombinant classes. Four (C1 and C3) or three (C2) recombinant classes were isolated. This is illustrated in the analysis of auxotrophic markers in Figure 2: 77 basidiospores germinated from the 80 manipulated from basidium C3. In the unlikely event that basidiospores could emanate from multiple rounds of meiosis, one expects a 1:1:1:1 ratio of *ade2 ura5*, *ADE2 URA5*, *ade2 URA5*, and *ADE2 ura5* progeny. Of the 77 progeny, 43 were *ADE2 URA5* and 34 were *ade2 ura5*, *i.e.*, all progeny were recombinants, since the parents are *ade2 URA5* and *ADE2 ura5*, and none of the progeny were *ade2 URA5* or *ADE2 ura5*.

The results of plating basidiospores from a single basidium and looking at two markers (Figure 2) demonstrate a single meiotic reduction per basidium; nevertheless, a more extensive genetic analysis of the events in the basidia was undertaken. For this purpose, a diploid *C. neoformans* strain was generated because such a strain provides a number of experimental benefits, and it was induced to sporulate on Murashige-Skoog medium. In a similar manner as described for regular crosses, as much of the ends of a basidium were attached to the dissecting microfilament, individual basidiospores were micromanipulated onto a YPD plate and allowed to germinate, and then the two auxotrophic markers and the mating type alleles were scored (Table 1). The technique is not particularly difficult, but one limitation was that it was rare to capture all four complete chains.

Including the three basidia analyzed from the conventional JF99 × M001 crosses, the basidiospores from 101 basidia were isolated, to yield 2,887 viable progeny (Table 1). The median germination of the spores per basidium was 90%, and the median number of viable progeny analyzed per basidium was 25. Analysis of the data presented in Table 1 shows a striking pattern: with only three exceptions (see discussion), all basidia yielded between two and four phenotypic classes. Collectively, these data support the reproductive model for *C. neoformans* of one meiosis followed by rounds of mitosis occurring in the basidium. The progeny derived from each basidium can be classified on the basis of marker segregation into parental ditypes (PD), nonparental ditypes (NPD), and tetratypes (TT), depending on chromosome assortment (Table 1). With three genetic markers, one expects 12 different combinations to occur (Bennett 1956). All 12 classes were detected (Table 2), supporting the use of each basidium as an unordered tetrad.

***ADE2* and *URA5* are linked to their centromeres:** For two unlinked markers in a tetrad analysis, the expected ratio resulting from random assortment in the progeny is 1 PD:1 NPD:4 TT. These ratios were examined in 94 basidia, excluding 7 basidia from the 101 total (14, 15, 42, 43, 44, 58, and 64) from subsequent analysis due to the inability to assign meiotic events or their unusual behavior. For *ADE2 URA5*, the proportions were 24 PD:36 NPD:34 TT. For *ADE2 MAT*, the proportions were 11 PD:18 NPD:65 TT. For *URA5 MAT*, the proportions were 15 PD:14 NPD:65 TT. *ADE2* and *URA5* exhibit a reduction in the proportion of tetratypes, and this is also seen in the skew in numbers in Table 2 with 43% of basidia generating either *ADE2 URA5* PDs or NPDs and *ADE2 MAT* TTs and *URA5 MAT* TTs. A reduction in the *ADE2 URA5* TT frequency indicates that the markers are close to the centromeres on their chromosomes, while the 2/3 TT frequency for *ADE2 MAT* and *URA5 MAT* implies that *MAT* is unlinked to its centromere. Having three markers in a cross can enable the location of the centromeres (*CEN*) on each chromosome to be mapped with a previously defined set of three formulas (Perkins 1949; Whitehouse 1950; Papazian 1952), but only when all three markers are centromere linked. The *ADE2 URA5* tetrad frequency of $34/94 = a + u - 3au/2$, where a and u are the second division segregation frequencies for *ADE2* and *URA5*. Using one marker not recombining with its centromere (a or $u = 0$) infers that the maximum distance for the other marker from its centromere is 18 cM.

TABLE 1

Phenotypes and numbers of each progeny type analyzed from individual basidia of *C. neoformans*

Basidium no.	Dissected	Germinated	Phenotypes *ADE2 URA5* α	*ADE2 URA5* **a**	*ADE2 ura5* α	*ADE2 ura5* **a**	*ade2 URA5* α	*ade2 URA5* **a**	*ade2 ura5* α	*ade2 ura5* **a**	*ADE2 URA5*	*ADE2 MAT*	*URA5 MAT*
C1	41	36	13	0	6	0	0	10	0	7	TT	NPD	TT
C2	32	22	7	0	0	0	7	0	0	8	TT	TT	PD
C3	80	77	21	22	0	0	0	0	16	18	NPD	TT	TT
1	23	21	4	0	4	0	0	5	0	8	TT	NPD	TT
2	34	33	9	0	10	0	0	6	0	8	TT	NPD	TT
3	16	14	4	4	0	0	0	0	5	1	NPD	TT	TT
4	28	26	6	1	0	0	0	0	10	9	NPD	TT	TT
5	20	15	4	7	0	0	0	0	0	4	NPD	TT	TT
6	21	20	0	0	1	3	7	9	0	0	PD	TT	TT
7	16	13	2	7	0	0	0	0	4	0	NPD	TT	TT
8	20	18	0	0	5	6	3	4	0	0	PD	TT	TT
9	19	18	4	2	0	0	0	0	7	5	NPD	TT	TT
10	24	23	6	6	0	0	0	0	6	5	NPD	TT	TT
11	15	14	2	0	0	4	5	0	0	3	TT	TT	PD
12	23	21	11	0	0	0	0	0	0	10	NPD	NPD	PD
13	14	14	0	0	3	3	4	4	0	0	PD	TT	TT
14	15	9	0	0	5	0	4	0	0	0	?	?	?
15	21	9	0	4	0	0	0	0	0	5	?	?	?
16	18	12	0	0	0	4	8	0	0	0	PD	PD	PD
17	21	20	11	0	0	0	0	0	0	9	NPD	NPD	PD
18	16	16	7	3	0	0	0	0	2	4	NPD	TT	TT
19	25	23	0	7	6	0	7	0	0	3	TT	TT	TT
20	29	15	6	0	0	0	0	0	0	9	NPD	NPD	PD
21	30	21	8	0	5	1	0	5	2	0	TT	TT	TT
22	38	37	0	18	0	0	0	0	19	0	NPD	PD	NPD
23	31	28	0	0	0	15	13	0	0	0	PD	PD	PD
24	30	26	8	9	0	0	0	0	8	1	NPD	TT	TT
25	41	33	0	0	18	0	0	15	0	0	PD	NPD	NPD
26	26	24	4	0	0	9	0	5	6	0	TT	TT	TT
27	40	40	0	0	18	0	0	22	0	0	PD	NPD	NPD
28	61	54	10	0	0	25	0	14	5	0	TT	TT	TT
29	22	14	0	0	0	5	9	0	0	0	PD	PD	PD
30	24	16	0	0	0	6	8	0	0	2	TT	TT	PD
31	46	37	13	0	10	0	0	9	0	5	TT	NPD	TT
32	22	19	0	6	4	0	5	0	0	4	TT	TT	TT
33	22	17	14	0	0	0	0	0	0	3	NPD	NPD	PD
34	18	15	0	0	2	3	6	4	0	0	PD	TT	TT
35	19	12	3	3	0	0	0	0	3	3	NPD	TT	TT
36	30	27	0	4	7	0	0	8	8	0	TT	TT	NPD
37	34	31	6	12	0	0	0	0	9	4	NPD	TT	TT
38	28	28	0	0	7	9	6	6	0	0	PD	TT	TT
39	24	24	0	0	2	16	4	2	0	0	PD	TT	TT
40	22	18	11	0	0	0	0	0	0	7	NPD	NPD	PD
41	23	17	0	7	6	0	4	0	0	0	TT	TT	TT
42	37	35	0	0	0	25	2	4	4	0	?	?	?
43	48	47	3	10	3	1	16	14	0	0	?	?	?
44	37	20	0	0	0	0	0	8	12	0	?	?	?
45	35	33	5	13	0	0	0	0	7	8	NPD	TT	TT
46	29	24	9	0	3	0	0	7	0	5	TT	NPD	TT
47	40	39	18	0	0	11	9	0	0	1	TT	TT	PD
48	24	20	3	5	0	0	0	0	10	2	NPD	TT	TT
49	31	28	0	0	15	1	6	6	0	0	PD	TT	TT
50	43	41	0	0	12	0	0	29	0	0	PD	NPD	NPD
51	30	22	0	0	7	0	10	5	0	0	PD	TT	TT

(*continued*)

TABLE 1

(Continued)

			Phenotypes										
Basidium no.	Dissected	Germinated	*ADE2 URA5* α	*ADE2 URA5* **a**	*ADE2 ura5* α	*ADE2 ura5* **a**	*ade2 URA5* α	*ade2 URA5* **a**	*ade2 ura5* α	*ade2 ura5* **a**	*ADE2 URA5*	*ADE2 MAT*	*URA5 MAT*
52	22	20	0	9	0	5	3	0	3	0	TT	PD	TT
53	70	68	16	0	0	18	18	0	0	16	TT	TT	PD
54	25	16	4	0	0	0	0	0	4	8	NPD	TT	TT
55	27	19	0	4	9	0	0	5	1	0	TT	TT	NPD
56	51	50	0	13	16	0	0	8	13	0	TT	TT	NPD
57	42	41	0	0	20	11	2	8	0	0	PD	TT	TT
58	67	46	(See text and Figure 3)								?	?	?
59	26	25	7	6	0	0	0	0	7	5	NPD	TT	TT
60	40	30	0	0	0	12	18	0	0	0	PD	PD	PD
61	62	52	27	0	0	0	0	0	0	25	NPD	NPD	PD
62	30	24	0	5	0	0	0	0	7	12	NPD	TT	TT
63	46	45	11	10	0	0	0	0	11	13	NPD	TT	TT
64	43	35	8	6	1	4	4	3	4	5	?	?	?
65	44	43	2	26	0	0	0	0	13	2	NPD	TT	TT
66	44	30	7	7	0	0	0	0	10	6	NPD	TT	TT
67	45	33	0	19	10	0	4	0	0	0	TT	TT	TT
68	28	26	1	0	11	12	0	2	0	0	PD	TT	TT
69	31	31	8	11	0	0	0	0	5	7	NPD	TT	TT
70	48	48	0	24	0	0	0	0	24	0	NPD	PD	NPD
71	23	23	0	5	5	0	10	0	0	3	TT	TT	TT
72	15	15	2	0	0	7	0	2	4	0	TT	TT	TT
73	44	40	10	0	0	25	0	3	2	0	TT	TT	TT
74	38	37	0	9	0	13	7	0	8	0	TT	PD	TT
75	38	36	12	8	0	0	0	0	8	8	NPD	TT	TT
76	24	18	0	6	0	6	0	0	6	0	TT	PD	TT
77	73	69	16	0	17	0	0	5	0	31	TT	NPD	TT
78	50	43	0	24	0	0	0	0	19	0	NPD	PD	NPD
79	42	30	0	14	0	0	0	0	16	0	NPD	PD	NPD
80	35	30	0	0	13	0	0	17	0	0	PD	NPD	NPD
81	42	37	0	0	11	10	7	9	0	0	PD	TT	TT
82	54	51	0	14	11	0	0	11	15	0	TT	TT	NPD
83	43	40	0	0	10	11	9	10	0	0	PD	TT	TT
84	46	42	0	11	9	0	0	8	14	0	TT	TT	NPD
85	36	17	9	0	0	0	0	0	5	3	NPD	TT	TT
86	55	53	0	0	14	15	11	13	0	0	PD	TT	TT
87	24	19	0	0	7	1	6	5	0	0	PD	TT	TT
88	25	15	4	5	0	0	0	0	0	6	NPD	TT	TT
89	37	23	6	6	0	0	0	0	5	6	NPD	TT	TT
90	41	20	10	0	0	0	0	0	4	6	NPD	TT	TT
91	27	20	6	0	6	0	0	6	0	2	TT	NPD	TT
92	42	19	0	0	0	0	9	10	0	0	PD	TT	TT
93	84	83	18	0	0	22	0	25	18	0	TT	TT	TT
94	30	28	0	6	8	0	6	0	0	8	TT	TT	TT
95	50	44	0	6	12	0	0	14	12	0	TT	TT	NPD
96	31	29	6	0	7	0	0	5	0	11	TT	NPD	TT
97	27	25	0	0	10	6	2	7	0	0	PD	TT	TT
98	26	21	14	4	0	0	0	0	2	1	NPD	TT	TT
Total	3376	2887	416	408	360	325	252	367	383	330			

C1–C3 are derived from JF99 × M001 crosses. Numbers 1–98 are derived from sporulation of diploid strain AI187. *ADE2 ura5* **a** and *ade2 URA5* α are the parental classes. Tetrads that cannot be classified are designated by (?).

C. neoformans is predicted to have centromeres composed of multiple repeated sequences with similarities to transposable elements, and within the genome there is a single repetitive element section per chromosome (Loftus *et al.* 2005). Data from a genomewide genetic map constructed from a cross between two serotype D parents (Marra *et al.* 2004) was used to estimate the recombination frequencies between genetic markers

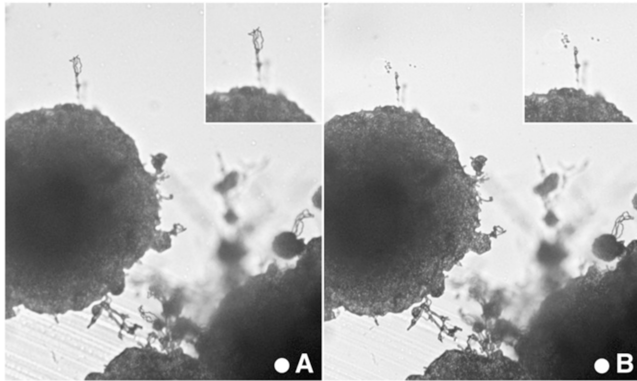

Figure 1.—Removing basidiospores from a basidium of *C. neoformans*. (A) Yeast cells and sporulation (inset) of the diploid strain AI187 prior to spore removal. (B) Image after using the microfilament to remove chains, in which several basidiospores remain near the basidium. The image resolutions are low because they are photographed through 6 mm of Murashige-Skoog 4% agar medium and the petri dish surface. White circles indicate the size (25 μm diameter) of the microfilament used to remove the chains of spores.

close to the predicted centromeres and the three markers used here. This approach could establish whether the linkage relationships defined from the tetrad analysis are consistent with the centromeres predicted on each chromosome. Sequences of the PCR–RFLP primers used in creating the genetic map of *C. neoformans* (Marra *et al.* 2004) were used in BLAST analysis against the JEC21 genome to identify markers flanking the *ADE2* and *URA5* genes, *MAT*, and the predicted centromeres, and the genetic segregation data from the map used for centimorgan distances. One caveat is that the genetic map was made with different strains than those used in this analysis and that recombination frequencies will likely vary between serotypes. However, it is known from genomewide comparisons that both serotype A and D genomes are largely syntenic, and specifically that no major chromosome arrangements affect the chromosomes on which *ADE2*, *URA5*, and the mating type loci reside (Kavanaugh *et al.* 2006; Sun and Xu 2009).

The mating type locus and associated genetic markers lie at position 68.5 cM of linkage group 20 on chromosome 4. The predicted centromere lies at the opposite end of the chromosome. No markers are close to the predicted centromere, but it must reside beyond position 0 cM of linkage group 20. Thus, that *MAT* is unlinked to its centromere, as defined through tetrad analysis in serotype A, is in agreement with the centromere being at the end of the chromosome. For the chromosome containing *ADE2*, the predicted centromere lies between genetic markers Xho7 and Hae4 (no recombination between them; position 102.8 cM) while *ADE2* lies between markers Pst22 (76.2 cM) and AGC9 (85.5 cM) of linkage group 7 on chromosome 5. On the basis of this information, the *ADE2–CEN* distance would be between 17.3–26.6 cM, *i.e.*, near the estimate based on mapping in serotype A tetrads. Similarly, for the chromosome containing *URA5*, the predicted centromere lies between markers Pst14 (position 69.8 cM) and Bam12 (72.9 cM), and *URA5* between markers Bam1 (86.7 cM) and GGC13 (95.0 cM) of linkage group 11 on chromosome 7. This would make the predicted *URA5–CEN* linkage between 13.8–25.2 cM, also close to the estimate from the tetrad analysis. While mapping the centromeres on these three chromosomes cannot define them as repetitive regions in the genome without the use of additional crosses and markers, the *ADE2–CEN*, *URA5–CEN*, and *MAT–CEN* distances are consistent with the hypothesis that these repeat-rich DNA regions could define the centromeres of *C. neoformans*.

Irregular nuclear reduction occurs in a subset of basidia to reduce the frequency of basidiospore germination: In the ideal situation the entire contents of a basidium could be removed and plated, resulting in progeny classes that form in exact ratios of 1:1 or 1:1:1:1. For some basidia this was nearly achieved. However, for others a proportion of the basidiospores, including parts of chains, was left behind. The proportions of each class in Table 1 show large skews for some basidia. While this could be accounted for by sample size, in many of these situations the germination frequency was also reduced, and the trend was a loss of one (or two) of the predicted classes of progeny (see basidia C2, 5, 30, 41, 51, 54, 67, 76, 85, 88, 90, and 92). It is thus possible that one of the original nuclei after meiosis has a major

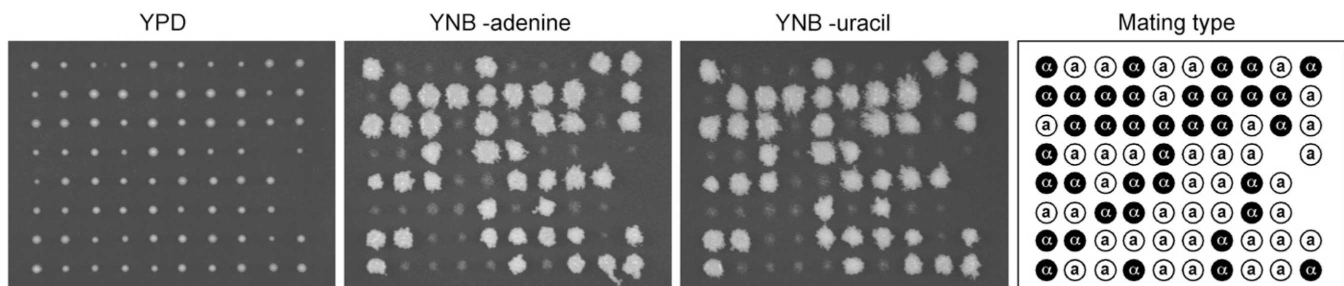

Figure 2.—Analysis of progeny from one basidium from a *C. neoformans* cross between *ade2 MAT*α × *ura5 MAT***a** strains showing one meiotic reduction per basidium. A total of 80 progeny were isolated from a single basidium (C3) and plated 5 mm apart on YPD agar. The 77 spores that germinated resulted in colonies that were replica plated to media to test for the *ADE2* and *URA5* alleles. The progeny were backcrossed to *MAT***a** and *MAT*α strains to determine mating type.

TABLE 2

Frequency of each tetrad class identified from analysis of the progeny in Table 1

ADE2 URA5	*ADE2 MAT*	*URA5 MAT*	Total (94)
PD	PD	PD	4
PD	TT	TT	16
TT	PD	TT	3
TT	TT	PD	5
NPD	TT	TT	26
TT	NPD	TT	8
TT	TT	NPD	6
PD	NPD	NPD	4
NPD	PD	NPD	4
NPD	NPD	PD	6
TT	TT	TT	7
TT	TT	TT	5

genetic impairment, and that all basidiospores derived from it are inviable.

The hypothesis that the spores that do not germinate originate from chromosome nondisjunction is supported by analysis of basidium 58. A subset of the basidiospores were aneuploid, and presumably another one-quarter of the progeny representing those in which the nucleus was missing a chromosome(s) were inviable (Figure 3). Of the 67 basidiospores that were isolated, 46 germinated (69%), which is lower than the median 90% and consistent with one-quarter of the progeny not germinating. This quarter would be derived from the initial meiotic reduction to generate four nuclei, three with the wild type or +1 chromosome complement that enters spores that germinate and are viable, and one with −1 chromosome that enters spores that do not germinate. Fifteen colonies were *ADE2 URA5 MAT***a**, 16 colonies were *ade2 ura5 MAT*α, and 15 colonies were *ADE2 URA5* and infertile in that the **a** or α phenotype could not be scored by crossing these strains with the *MAT***a** and *MAT*α testers. This infertile phenotype triggered a PCR-based investigation of alleles in these progeny. Analysis of the *URA5* allele (via a *Cla*I site polymorphism between the wild-type and mutant *ura5* allele of JF99) and the *ADE2* gene (via amplification of the gene by PCR and DNA sequencing) revealed that the strains with wild-type growth contained only the wild-type allele. That is, the Ade+ Ura+ strains most likely have a single copy of each of these chromosomes, although the possibility of disomic chromosomes for each cannot be excluded. In contrast, analysis of the *MAT***a** and *MAT*α alleles (as determined by PCR specific to each allele using primers within the mating type locus) revealed that the 15 infertile *ADE2 URA5* strains contained both *MAT***a** and *MAT*α alleles, suggesting these strains contain two copies of the chromosome bearing the *MAT* locus, and are thus aneuploid (Figure 3). For basidium 58, it can be hypothesized that of the 21 basidiospores that did not germinate, most did not

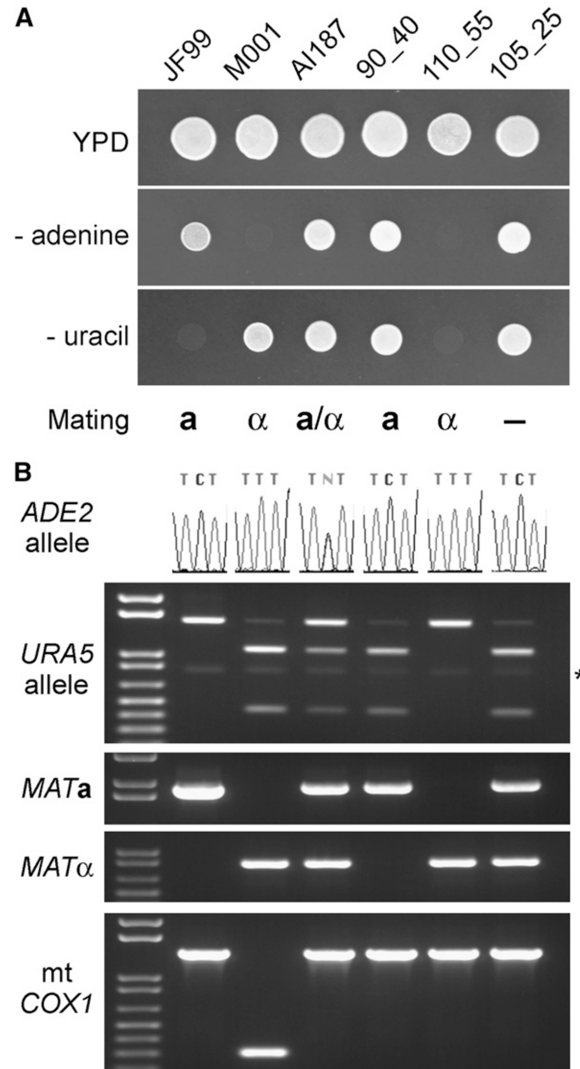

FIGURE 3.—Aneuploid strains can be isolated from basidia. Phenotypes (A) and genotypes (B) of the parental strains JF99 and M001, diploid AI187, and representatives of three phenotype classes derived from basidium 58 (90_40, 110_55, and 105_25). Yeast cultures were plated onto rich media (YPD) or YNB supplemented to test for growth in the absence of adenine or uracil. Genotypes were assigned by PCR amplification of each allele and resolution on 1% agarose gels, and in the case of *ADE2* by DNA sequence analysis. For the diploid AI187, the middle nucleotide was an equal mix of C and T. For *URA5*, the * marks a nonspecific amplification product. The mitochondrial genome gene *COX1* shows inheritance from the *MAT***a** JF99 parent in the diploid strain. The first lane in each gel is a DNA standard (Invitrogen 1 kb+ ladder).

because they contained a nucleus missing the *MAT* chromosome and the ∼600 genes located on it.

Chains are composed of basidiospores with multiple phenotypes: Another potential source for variation in numbers of each class could be the inability to isolate all the spores from all four chains. For example, occasionally a large proportion of one chain was left behind when the microfilament was brought close to the basidium. If all the progeny in a chain are of one phenotypic class,

TABLE 3

Phenotypes and numbers of each progeny type analyzed from individual chains isolated from basidia of *C. neoformans*

			Phenotypes										
Chain no.	Dissected	Germinated	*ADE2 URA5* α	*ADE2 URA5* **a**	*ADE2 ura5* α	*ADE2 ura5* **a**	*ade2 URA5* α	*ade2 URA5* **a**	*ade2 ura5* α	*ade2 ura5* **a**	*ADE2 URA5*	*ADE2 MAT*	*URA5 MAT*
1	6	6	4	0	0	0	0	0	0	2	NPD	NPD	PD
2	10	7	3	2	0	0	0	0	2	0	NPD	TT	TT
3	8	6	0	0	2	4	0	0	0	0	PD	TT	TT
4	7	7	5	0	2	0	0	0	0	0	TT	NPD	TT
5	7	4	0	0	3	0	0	0	0	1	TT	NPD	TT
6	18	13	0	0	3	4	5	1	0	0	PD	TT	TT
7	6	6	0	0	1	2	1	2	0	0	PD	TT	TT
8	5	5	0	0	0	1	3	0	1	0	TT	PD	TT
9	12	12	0	1	0	0	0	0	3	8	NPD	TT	TT
Total	79	66	12	3	11	11	9	3	6	11			

All chains originate from sporulation of diploid strain AI187. Chain 7 was part of basidium 81 (Table 1).

then this could skew the data. To test whether chains are composed of one or more spore types, individual chains of spores were isolated and the resulting progeny analyzed. Two previous experiments analyzing spore chains provided conflicting findings about whether a chain is made up of one type of progeny or multiple types (Kwon-Chung 1980; Kline and Jacobson 1981). Here, the fragments of nine chains from different basidia were isolated (Table 3 and Figure 4). In all nine situations two to four progeny classes could be isolated, demonstrating that the spore chains contain mixed genotypes.

Chains of basidiospores show differences in the degree of genetic heterogeneity, as illustrated by comparing chain 7 and chain 9. Chain 7 was isolated during the analysis of basidium 81. By chance, a fragment of six attached basidiospores was plated and then subdivided into individual basidiospores. During this division, two pairs of basidiospores remained attached, enabling the phenotypes of adjacent spores on a chain to be determined. In this example, all four phenotypes were found and were the same as found in the large data set of basidium 81. The pairs of spores in both instances were heterogeneous. In contrast, of the 12 progeny of chain 9, eight (66%) were of one genotype (*ade2 ura5 MAT***a**), three were *ade2 ura5 MAT*α, and one was *ADE2 URA5 MAT***a**. A fourth class (*ADE2 URA5 MAT*α) was absent, which is a significant difference from chance ($P = 0.75^{12} = 0.032$) of the expected 25% for this progeny.

DISCUSSION

The sexual cycle of *C. neoformans* has been studied for more than three decades, yet many aspects about the role of mating in the biology of this fungus remain to be resolved. One key example is a lack of knowledge about the events occurring in the basidium that result in nuclear reduction and the production of chains of spores, and the focus of this investigation. The ideal approach to examine spore production is a genetic one, *i.e.*, by analyzing genetic markers in progeny derived from a single basidium. Just such an approach can be achieved by careful micromanipulation of the spores from the

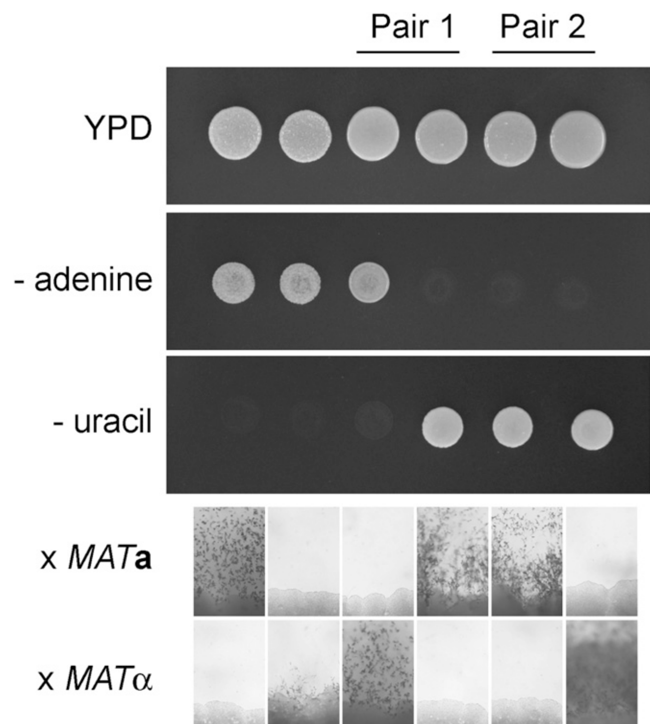

Figure 4.—Basidiospore chains of *C. neoformans* contain a mixture of genotypes. Six spores attached as a chain were micromanipulated (from basidium 81; chain 7 in Table 3). Initially the six broke into two individual spores and two pairs that were subsequently separated. Auxotrophic markers were scored on selective media and mating type scored by the production of filaments with the opposite mating partner (the dark mass in the photographs taken of the edges of colonies; each panel width ~1 mm).

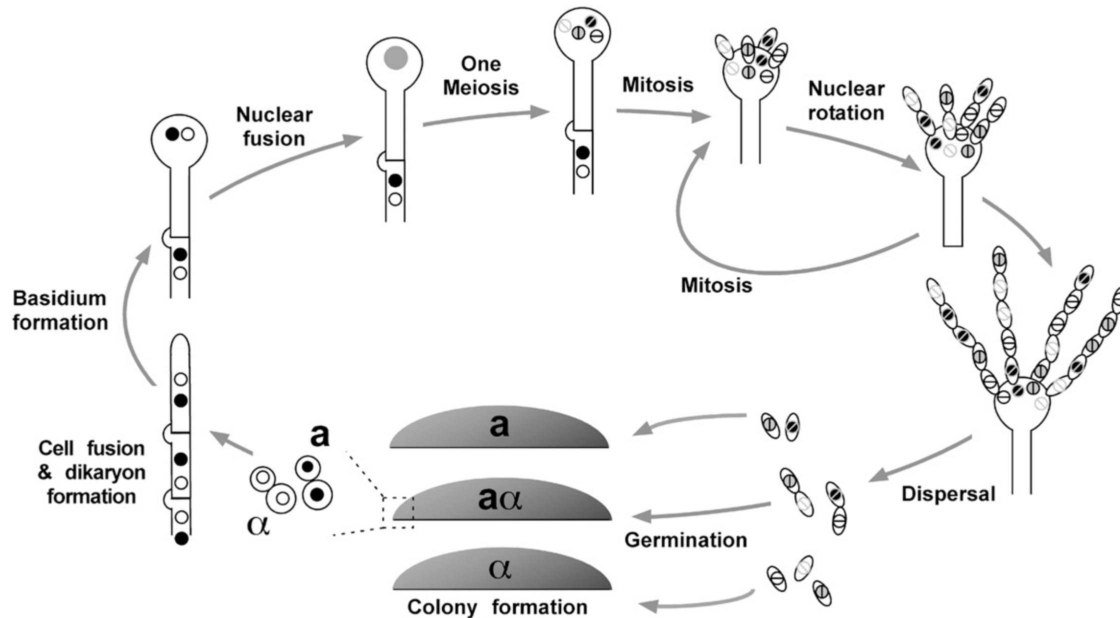

Figure 5.—A revised model of the *C. neoformans* life cycle, including the generation of heterogeneous chains and the attachment of basidiospores during dispersal that enables establishment of a genetically diverse new population. The nuclei are marked to indicate specific genotypes or changes after meiosis. The life cycle is based on that of Lin (2009).

ends of basidia, and in this study the nature of the reductive event in the basidium is defined genetically by taking whole basidia and analyzing the phenotypes of all progeny.

Spores derived from 101 individual basidia were isolated and their phenotypes determined (Table 1). The data can be analyzed and interpreted in a number of ways: the interpretation placed here is that in a *C. neoformans* basidium there is a single meiosis or other type of nuclear reduction event and multiple rounds of mitosis to generate the nuclei that are packaged into the basidiospores. This is supported by the observation that of the 101 basidia analyzed, 98 had four or less classes of progeny. Of the remaining three, one had a single additional genotype (21) while two (43 and #64) may have been derived from mixed chains. Basidium 42 has three *ade2* phenotype classes, although this could be explained by gene conversion. It is possible that these represent errors that occurred during the manipulation of nearly 3000 strains, in taking chains from more than one basidium where they overlapped rather than a single set of four chains, or in assigning and annotating the mating types of each strain.

One puzzling and frustrating aspect of genetic analysis in *C. neoformans* is why basidiospores do not exhibit 100% germination. One proposition (*e.g.*, see discussion in Velagapudi *et al.* 2009) is that the spores that do not germinate are dormant. However, analysis of individual basidia suggests that the spores are more likely to be aneuploid and thereby inviable, rather than dormant. In 12 examples the progeny are missing one or two genetic classes of the tetrad, and this includes one set from basidia from the JF99 × M001 cross, so cannot be attributed to instability of the genome in the diploid AI187 strain. Thus, it could be that one of the nuclei generated after meiosis is damaged or missing a chromosome, and the spores packaged with it are unable to germinate. Basidium 58 shows low germination and has three types of progeny that include 1/3 being aneuploid containing both alleles for *MAT* (Figure 3). Because the parental strains used here are largely congenic, it was not possible to use additional genetic markers to further explore this hypothesis: studies using diverged strains, or comparative genome hybridization, would be informative.

Two genetic analyses have studied chains of spores and are related to the technique reported here. In the first, three individual chains of basidiospores were dissected and two markers analyzed in the resultant progeny to show that the chains contained a mixture of phenotypes (Kwon-Chung 1980). In the second, chains primarily composed of four to six basidiospores were analyzed (Kline and Jacobson 1981). The entire chains were placed on the agar surface and allowed to germinate, and then eight individual colonies derived from each colony were analyzed with one genetic marker (Nia). Eleven of the chains were homogenous while the other three contained both Nia^+ and Nia^- spores. Here, isolation of individual chains and analysis of their basidiospores demonstrate that the chains are a heterogeneous population of spores. Nuclear movement in the basidium has been previously proposed to enable chains to be composed of spores with different genotypes (Kwon-Chung 1980). The rotation of nuclei in the basidium could be through either a ratchet or stochastic system. The stochastic model is preferred on the basis of

these data because it can explain the variation seen in phenotype distribution between different spore chains and the seemingly conflicting conclusions reached in the two previously published spore chain studies (Kwon-Chung 1980; Kline and Jacobson 1981). The nuclei can move after each mitosis, but can also remain fixed under the basidium position from which the spores bud, generating sections of identical phenotypes and then move again. The rate of movement could be controlled by environmental factors such as temperature.

What evolutionary benefit would there be to an organism, such as *C. neoformans*, in mating to produce chains of attached spores, and generating them via rounds of mitosis? One can only speculate as to why the species would not follow the approach taken by the highly successful mushroom, rust, or smut species that produce basidia with four spores. Two aspects of *C. neoformans* mating provide it the advantages of both genetic diversity and dispersal (Figure 5). First, basidiospores adhere to their neighbors along the chains and could be dispersed as a unit or partial unit: evidence for this attachment is both in the ability to isolate chains of attached spores and the occasional requirement during regular random spore analysis to have to use mechanical force to separate pairs of spores. Second, the proposed rotation of the nuclei within the basidium results in chains made up of different meiotic classes. Together, this provides an effective means of both generating large numbers of spores and spreading two or more genotypes at a time if part of a chain is moved as a unit (Figure 5). The resulting yeast colony will comprise more than one genotype, often including both mating type alleles enabling subsequent mating to occur. The mechanism is particularly useful for a yeast species that has a restricted ability to spread by growing, in contrast to filamentous fungal species. Mating of *C. neoformans* remains to be characterized in the wild, as are the vectors for basidiospore dispersal, and as such, environmental sampling is key to addressing the current life cycle model and further understanding the sexual biology of this organism and its relatives.

I thank Xiaorong Lin and Michael Plamann for comments on drafts of the manuscript, Aaron Mitchell for editorial and genetic suggestions, and Victor Laronga (Micro Video Instruments, Avon, MA) for assistance in locating parts for the microscope. This research was supported by a grant from the University of Missouri Research Board and National Institutes of Health K22 award AI073917.

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Communicating editor: A. P. Mitchell