

Development and Characterization of a Genetic Linkage Map of *Cryptococcus neoformans* var. *neoformans* Using Amplified Fragment Length Polymorphisms and Other Markers

Anja Forche,^{*†1} Jianping Xu,^{*†} Rytas Vilgalys,^{*} and Thomas G. Mitchell[†]

^{*}Department of Biology, Duke University, Durham, North Carolina 27708; and [†]Department of Microbiology, Duke University Medical Center, Durham, North Carolina 27710

Accepted for publication November 29, 2000

Forche, A., Xu, J., Vilgalys, R., and Mitchell, T. G. 2000. Development and Characterization of a Genetic Linkage Map of *Cryptococcus neoformans* var. *neoformans* Using Amplified Fragment Length Polymorphisms and Other Markers. *Fungal Genetics and Biology* 31, 189–203. A segregating population of single basidiospore isolates from a sexual cross was used to generate the first moderately dense genetic linkage map of *Cryptococcus neoformans* var. *neoformans* (Serotype D). Polymorphic DNA markers were developed using amplified fragment length polymorphisms, random amplified polymorphic DNA, and gene-encoding sequences. These markers were used to analyze 100 meiotic progeny. All markers were tested for distorted segregation with a goodness of fit test. Of the total of 181 markers, 148 showed balanced (1:1) segregation ratios. Segregation distortion was observed for 33 markers. Based on all the markers, a linkage map was generated that consists of 14 major linkage groups with 127 markers, several small linkage groups, and 2 linkage groups that consist only of highly skewed markers. The genetic distance of the linkage map is 1356.3 cM. The estimated total haploid genome size for *C. neoformans* var. *neoformans* was calculated using Hulberts method and yielded a map

size of 1917 cM. The number of major linkage groups correlates well with the proposed number of 13 chromosomes for *C. neoformans* var. *neoformans*. Several genes, including *CAP64*, *CnLAC*, and the mating-type locus, were mapped, and their associations were consistent with published data. To date, 6 linkage groups have been assigned to their corresponding chromosomes. This linkage map should provide a framework for the ongoing genome sequencing project and will be a useful tool for studying the genetics and pathogenicity of this important medical yeast. © 2000 Academic Press

Index Descriptors: *Cryptococcus neoformans*; genetic linkage map; amplified fragment length polymorphism (AFLP); karyotyping.

Cryptococcus neoformans is a haploid basidiomycetous yeast. A major phenotypic feature of *C. neoformans* is its thick enveloping polysaccharide capsule. Antigenic differences among the capsules of strains were used to identify five serotypes—A, D, AD, B, and C. Three varieties of *C. neoformans* have been recognized. *C. neoformans* var. *gattii* is represented by capsular serotypes B and C and appears to be geographically limited to tropical regions. Serotypes A, D, and AD are found globally and are associated with two closely related varieties, *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A) (Xu *et al.*, 2000; Casadevall and Perfect, 1999; Franzot *et al.*, 1999). The mode of reproduc-

¹ To whom correspondence should be addressed at current address: Department of Genetics, Cell Biology and Development, University of Minnesota, St. Paul, MN 55108. Fax: (612)-625-5754. E-mail: forche@mail.ahc.umn.edu.

tion of all serotypes and varieties is primarily asexual, but in the laboratory, compatible strains of opposite mating types can be induced to engage in sexual reproduction (Kwon-Chung, 1976). Mating compatibility is governed by one locus with two alleles, denoting mating type a or α . Under laboratory conditions, sexual crosses can be obtained between strains of the opposite mating type and between the same or different serotype.

In the past decade, *C. neoformans* has emerged as a human pathogen of life-threatening consequences, especially in immunocompromised patients. Indeed, cryptococcal meningoencephalitis is the leading mycological cause of death among patients with AIDS. Several properties of *C. neoformans* have been associated with its virulence, including the capsule, production of melanin, and the α mating type (Casadevall and Perfect, 1999; Kwon-Chung and Rhodes, 1986). Cryptococcosis can be treated with native or liposomal amphotericin B, but AIDS patients with cryptococcal meningitis require life-long maintenance therapy with fluconazole. Since relapses are common and drug-resistant strains have been reported (Casadevall and Perfect, 1999), better antifungal drugs are being sought.

A genetic linkage map of *C. neoformans* will foster understanding of the genetic organization of this important pathogen, enable important genes to be mapped, and support the genome project to sequence *C. neoformans*. The search for better understanding of phenotypically important traits, including drug resistance, would also benefit from a genetic linkage map of *C. neoformans*. Because of its *in vitro* mating characteristics, the current genome effort, and its clinical importance, *C. neoformans* is an ideal pathogenic fungus for a genetic linkage map. The initial efforts to study the genetic organization of a genome entail the development of physical and genetic linkage maps.

Little is known about the genome and the genome organization of *C. neoformans*. Several karyotyping studies have been used to develop rudimentary physical maps of *C. neoformans* (Perfect *et al.*, 1989; Polacheck and Lebens, 1989; Spitzer and Spitzer, 1997; Wickes *et al.*, 1994). Chromosome (CH)-specific probes have been developed for *C. neoformans* (Spitzer and Spitzer, 1997; Wickes *et al.*, 1994). Wickes *et al.* (1994) located 10 genes on chromosomes that were separated by pulsed-field gel electrophoresis (PFGE) and transferred to a nylon membrane; representatives of four serotypes (A, B, C, and D) were evaluated, and linked genes were identified on seven chromosomes (Wickes *et al.*, 1994). Electrophoretic karyotyping has also been used to identify strains in epidemiological studies of *C. neoformans* (Boekhout and van Belkum, 1997; Boekhout *et al.*, 1997; Thompson *et al.*, 1999). For clinical and environmental strains of the four major sero-

types, karyotyping offered a high level of resolution and discrimination for epidemiological studies. Spitzer and Spitzer (1997) hybridized partial sequences of randomly chosen cDNA clones to blots of electrophoretic karyotypes produced by PAGE. They identified at least seven chromosomes of *C. neoformans* strain B3501. These physical maps should complement the genetic linkage map. Another group performed a recombinational mapping analysis of capsular mutants (Still and Jacobson, 1983); pairs of capsule-negative mutants were crossed, and random spore analysis determined the proportion of wild-type recombinants. In this investigation, all seven capsule-negative mutants were linked within a distance of 35 map units.

For this emerging pathogen, a combined physical and genetic linkage map has not been developed. The goals of this study were to (i) develop genetic markers, (ii) produce a linkage map based on these markers, (iii) increase understanding of the genetics and basic biology of *C. neoformans*, (iv) examine meiotic behavior, which has not been extensively studied, and (v) provide a framework for future genetic analyses and genomics of *C. neoformans*. To generate a genetic linkage map of *C. neoformans* var. *neoformans*, we used amplified fragment length polymorphisms (AFLP), random amplified polymorphic DNAs (RAPDs), and gene-encoding regions. Using selected markers for hybridization studies, linkage groups were physically located on the appropriate chromosome. In addition to abetting the *C. neoformans* genome sequencing project, this genetic linkage map should provide a powerful tool for researchers to investigate the genetics and molecular pathogenesis of *C. neoformans*. To generate basidiospores, we mated two strains that were originally derived from a cross between a clinical isolate, NIH412, and an environmental strain, NIH433 (Heitman *et al.*, 1999). The original MAT α parent (NIH412) was self-fertile, but neither F₁ progeny (B-3501 and B-3502) posed this problem. The electrophoretic karyotypes of these mating strains are quite dissimilar, which suggests the likelihood of significant genetic polymorphism between them and therefore that they are good strains to select for the construction of a genetic map (Heitman *et al.*, 1999).

MATERIALS AND METHODS

Mating Experiment

Two strains of *C. neoformans* serotype D, B3502 (mating type a or MAT a) and B3501 (MAT α), were crossed on V8 juice agar (Xu *et al.*, 2000), and 280 random basidios-

TABLE 1
Adapter and Primer Sequences for the *MseI* and *EcoRI* Restriction Sites

	Restriction site	Primer	Sequence	
Adapter	<i>MseI</i>		5'-GACGATGAGTCCTGAG-3'	
Universal primer			3'-TACTCAGGACTCAT-5'	
Preamplification		M	5'-GATGAGTCCTGAGTAAA-3'	
		Ma	5'-GATGAGTCCTGAGTAAAA-3'	
		Mc	5'-GATGAGTCCTGAGTAAAC-3'	
		Mg	5'-GATGAGTCCTGAGTAAAG-3'	
		Mt	5'-GATGAGTCCTGAGTAAAT-3'	
		Selective amplification	Mac	5'-GATGAGTCCTGAGTAAAA C -3'
			Mga	5'-GATGAGTCCTGAGTAAAA GA -3'
			Mgt	5'-GATGAGTCCTGAGTAAAG T -3'
			Mtg	5'-GATGAGTCCTGAGTAAAT G -3'
			Mtgc	5'-GATGAGTCCTGAGTAAAT G C -3'
Adapter			<i>EcoRI</i>	
				3'-CATCTGACGCATGGTTAA-5'
Universal primer	E	5'-GACTGCGTACCAATTCA-3'		
Preamplification	Ec	5'-GACTGCGTACCAATTCAC-3'		
	Eg	5'-GACTGCGTACCAATTCAG-3'		
	Et	5'-GACTGCGTACCAATTCAT-3'		
	Selective amplification	Eca		5'-GACTGCGTACCAATTCACA-3'
		Egt		5'-GACTGCGTACCAATTCAG T -3'
		Egtc		5'-GACTGCGTACCAATTCAG T C -3'
		Etg		5'-GACTGCGTACCAATTCAT G -3'

Note. Abbreviations of the primers are given. Nucleotide extensions for preamplification and selective amplification are indicated in boldface. *EcoRI* primers for selective amplification were labeled with fluorescent dye 6FAM (Egt, Egtc, Etg) or 9TET (Eca).

pores were selected and used for genetic analyses. To determine the mating type, each progeny was mated with both parents and to the congenic tester strains, JEC20 (MAT α) and JEC21 (MAT α). In this paper we refer to B3502 as parent A and to B3501 as parent B.

DNA Extraction

DNA was extracted according to an established procedure (Xu *et al.*, 2000).

Development of AFLP Markers

Digestion of genomic DNA with restriction endonucleases (REs). To initially evaluate the REs planned for the AFLP protocol (Vos *et al.*, 1995), genomic DNA of *C. neoformans* was digested with two REs, *EcoRI* and *MseI*. One hundred fifty nanograms of genomic DNA was mixed in a final volume of 40 μ l containing 100 mM Tris/acetic acid (pH 7.5), 100 mM magnesium acetate, 500 mM potassium acetate, 50 mM dithiothreitol (DTT), 0.5 mg/ml bovine serum albumin (BSA), and 5 units each of *EcoRI* and *MseI* and incubated at 37°C for 2.5 h. As a control for complete digestion by the REs, 50 ng of lambda DNA was subjected to treatment with 10 μ l of

each RE. After digestion, DNA was examined by electrophoresis in 1.5% agarose. Complete digestion of the lambda DNA, as well as a lack of high-molecular-weight genomic DNA of *C. neoformans*, indicated that this treatment likely resulted in complete digestion.

Ligation of the *EcoRI* and *MseI* adapters to the restriction fragments. To each 40- μ l RE digestion tube, 10 μ l AFLP ligation cocktail, consisting of 100 mM Tris/acetic acid (pH 7.5), 100 mM magnesium acetate, 500 mM potassium acetate, 50 mM DTT, 0.5 mg/ml BSA, 100 mM ATP, 1 Weiss unit T4 ligase, 5 μ M double-stranded *EcoRI* adapters, and 50 μ M *MseI* adapters, was added. Reactions were incubated at room temperature (22°C) for 3 h. Each reaction was then diluted with 5–10 volumes of distilled water depending on the initial concentration of genomic DNA. The *EcoRI* and *MseI* adapters are listed in Table 1.

Preamplification of the AFLP markers. PCRs were performed in a total volume of 25 μ l with 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 100 mM each dATP, dCTP, dGTP, and dTTP (Amersham Pharmacia Biotech, New Jersey, USA), 10 μ M each primer (see Table 1 for primer sequences), 1.25 units *Taq* polymerase, and 5 μ l of the diluted ligation product. PCR conditions comprised 20 cycles with a denaturation step for 30 s at

Marker	ParentA (B3501)	ParentB (B3502)	Progeny Code
1	0	1	A (shared with parent A)
2	1	0	H (heterozygotic with parent A)

FIG. 1. Example of a scoring pattern for a backcross to parent A (B3501). The example shows two markers: one is absent in parent A and the other is present in parent A. The progeny strain shares marker 1 with parent A (scored as A) and is heterozygous for marker 2 (scored as H).

94°C, an annealing step for 1 min at 56°C, and an extension step of 1 min at 72°C. Preamplification PCR products were diluted 10-fold prior to selective amplification.

Selective amplification of the AFLP markers. PCRs were performed as above, except that the *EcoRI* primers were labeled with a fluorescent dye to detect AFLPs with the ABI 377 sequencer (Perkin–Elmer, Foster City, CA) and 5 μ l diluted PCR preamplification products served as DNA templates. For selective amplification, two and three nucleotides were added to the 3' end of the primers (see Table 1). PCRs were performed over 13 cycles with a denaturation step for 30 s at 94°C, an annealing step for 30 s at 65°C with a temperature decrease of 0.7°C per cycle, and an extension step of 1 min at 72°C, followed by 20 cycles with a denaturation step for 30 s at 94°C, an annealing step for 30 s at 56°C, and an extension step for 1 min at 72°C.

Analysis of AFLP patterns. A 5- μ l sample of each PCR product was electrophoresed in 1.5% agarose to confirm successful amplification and roughly quantify the PCR product by comparison with a 100-bp DNA molecular weight marker. AFLP samples were run on an ABI 377 sequencer (Perkin–Elmer, Norwalk, CO) for 4.5 h, and samples were analyzed using Genescan software, version 3.0 (Perkin–Elmer). The AFLP patterns of each strain were compared with the two parental strains, and all polymorphic markers, which were present in one parent and absent in the other, were scored. The 100 progeny strains were then scored for these polymorphic markers. The results were expressed as a binary data set, coded, and analyzed as a back cross to parent A (Fig. 1). Segregation values were calculated for each marker, and a simple goodness of fit test (χ^2 test) was applied to test for significance of segregation (i.e., any skewing from the expected Mendelian segregation ratio of 50:50).

Development and Analysis of RAPD Markers

To develop RAPD markers (Williams *et al.*, 1990), initially 67 pairwise combinations of arbitrary 10-mer oligo-

nucleotides (Operon Technologies Inc., Alameda, CA; Kits A and B) and 10 single 10-mer oligonucleotides (Operon Technologies Inc.; Kits A and B) were tested for polymorphisms between the parental strains, B3501 and B3502. Briefly, PCRs were carried out in a total volume of 25 μ l with 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 100 mM each dATP, dCTP, dGTP, and dTTP (Pharmacia), 4.5 mM magnesium acetate, 1.25 units *taq* polymerase, 10 ng genomic DNA, and a final primer concentration of 0.6 μ M each. PCR conditions comprised 45 cycles with a denaturation step for 1 min at 95°C, an annealing step for 1 min at 36°C, and an extension step of 2 min at 72°C. PCR products were electrophoresed on a 1.5% agarose gel with ethidium bromide in 1 \times TAE for 5 h at 4.3 V/cm. Primer combinations that revealed unambiguously scorable polymorphisms between the parental strains were tested for all 100 progeny. The sequences of these primers are listed in Table 2. The scoring of the RAPD markers was similar to that of the AFLP markers (see above).

Development and Analysis of Gene-Encoding DNA Markers

Fifteen different gene-encoding DNA sequences were tested for polymorphisms between the two parental strains, B3501 and B3502 (see Table 3). For 11 of the genes, sequences were obtained from GenBank, primer pairs were designed, and PCR conditions were optimized to amplify a single PCR fragment representing the correspondent DNA coding region. Primers that revealed polymorphisms between the parental strains and primer sequences are given in Table 2. PCRs were performed in a total volume of 25 μ l with 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 1.25 mM each dATP, dCTP, dGTP, and dTTP, 10 μ M each primer, 1.25 units *taq*, and 10–50 ng of genomic DNA. PCR amplifications were carried out over 35 cycles with an initial denaturation step at 94°C for 3 min, a denaturation step at 94°C for 1 min, an annealing step (specific temperature for each primer pair) for 30 s, an extension step at 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were purified using a Qiaquick PCR purification kit (Qiagen, Valencia, CA) and sequenced using a standard dye-terminator sequencing kit following the manufacturer's instructions (Applied Biosystems Inc.) with an automated sequencer (ABI Model 377 or 373; Perkin–Elmer). Sequence data were assembled and analyzed using Sequencer 3.0 software (Gene Codes Corp., Inc.). For DNA sequences that revealed polymorphisms between the parental strains, PCR products of all 100 progeny

TABLE 2
RAPD Primers and Primers to Amplify Gene-Encoding DNA Regions That Revealed Polymorphisms between the Parental Strains B3501 (Parent B) and B3502 (Parent A)

Primer	Name of primer	Primer sequence (5'-3')
RAPD		
	A1	CAGGCCCTTC
	A2	TGCCGAGCTG
	A7	GAAACGGGTG
	A8	GTGACGTAGG
	A9	GGTAACGCC
	A10	GTGATCGCAG
	A11	CAATCGCCGT
	A17	GACCGTTTGT
	B10	CGTCTGGGAC
	B11	GTAGACCCGT
	B15	GGAGGGTGTT
Gene-encoding DNA regions		
<i>CAP64</i>	CAP5	CCAAGGGAGTCTTATATGGCGAC
	CAP6	GTAATAGCCACATCGCCCGG
<i>CnLAC1</i>	LAC1	GGCGATACTATTATCGTA
	LAC2	TTCTGGAGTGGCTAGAGC
<i>URA5</i>	URA3	ACGCTGCCTGTTACTTAA
	URA4	GGACATGATGATTGGAGT
<i>Ste11</i>	Ste11-1	ATGGCAGCTTTACACCAGCCTTC
	Ste11-2	GATACCACCAATAAGCCTTACC
<i>Ste12</i>	Ste12-1	CTGAGGAATCTCAAACCGGG
	Ste12-2	CCAGGGCATCTAGAAACAATCG
<i>Ste20a</i>	Ste20a-1	GATCTGTCTCAGCAGCCAC
	Ste20a-2	AAAATCAGCTGCGCAGGTGA
<i>Ste20α</i>	Ste20 α -1	GATTTATCTCAGCAGCCACG
	Ste20 α -2	AAATCGGCTACGGGACGTC

Note. Abbreviations of the primer names are given and the primer sequences are arranged from the 5'-3' end.

strains were sequenced, polymorphisms were detected, and data sets were generated as described above (see Development of AFLP Markers). For the remaining four genes, *Ste11*, *Ste12*, *Ste20* MATa, and *Ste20* MAT α , polymorphisms were scored on the presence or absence of a single amplified PCR band for each gene. The amplification of *Ste11* and *Ste12* was performed as described above. *Ste20* MATa and *Ste20* MAT α were amplified in a total volume of 25 μ l with 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 100 mM each dATP, dCTP, dGTP, and dTTP, 10 μ M each primer, 1.25 units *taq* polymerase, and 10–50 ng of genomic DNA, respectively. PCRs were carried out over 25 cycles with an initial denaturation step at 94°C for 5 min, a denaturation step at 94°C for 30 s, an annealing step at 65°C for 30 s, an extension step at 72°C for 30 s, and a final extension step at 72°C for 5 min. Markers were scored as described.

Linkage and Mapping Analysis

Linkage and mapping analyses of the combined data sets were performed using MAPMAKER software, version 3.0 (Lander and Green, 1987; Lander *et al.*, 1987). To initially generate linkage groups, only markers with balanced segregation ratios (1:1) were analyzed. Linkage groups were formed using a likelihood of (log) odds (LOD) score of 5.0 and a recombination fraction of 0.2 (25.2 cM maximum distance [Haldane function] between two linked markers) (Morton, 1955). After stable linkage groups were obtained, more markers were added by stepwise relaxing of the recombination fraction value to 0.3 (approximately 48 cM maximum distance between two markers). Additionally, markers with skewed segregation ratios ($\chi^2 > 3.84$, $P < 0.05$) were added to the analysis and examined for linkage. To check for progeny strains with identical genotypes that might have derived from the same basidiospore isolate, UPGMA cluster analysis was carried out on the combined data set, revealing that all progeny strains had unique genotypes and differed from each parental genotype (data not shown).

Electrophoretic Karyotypes

To karyotype the two parental strains, pulsed field gel electrophoresis was performed following a published protocol (Wickes *et al.*, 1994). Briefly, four loopfuls of liquid culture were transferred to plates of yeast extract-potato dextrose agar or yeast morphology agar (Difco), and cells were grown at 30°C for up to 5 days. Five loopfuls of cells were then transferred onto yeast nitrogen broth (Difco)

TABLE 3
DNA Coding Regions That Were Tested for Polymorphisms between the Two Parental Strains B3501 (Parent A) and B3502 (Parent B)

No.	DNA coding region/gene	Citation
1	<i>Ste11</i>	J. Heitman <i>et al.</i> , unpublished
2	<i>Ste12</i>	Wickes <i>et al.</i> , 1997
3	<i>Ste20a</i>	Lengeler <i>et al.</i> , 2000
4	<i>Ste20α</i>	Sia <i>et al.</i> , 2000
5	<i>CAP59</i>	Chang and Kwon-Chung, 1994
6	<i>CAP64</i>	Chang <i>et al.</i> , 1996
7	<i>CNLAC1</i>	Williamson, 1994
8	<i>URA5</i>	Edman and Kwon-Chung, 1990
9	<i>HIS3</i>	Parker <i>et al.</i> , 1994
10	Small subunit <i>rRNA</i>	Hong <i>et al.</i> , 1993
11	<i>TDS</i>	Livi <i>et al.</i> , 1994
12	<i>TRP1</i>	Perfect <i>et al.</i> , 1992
13	<i>UBC</i>	Spitzer and Spitzer, 1995
14	<i>DHFR</i>	Sirawaraporn <i>et al.</i> , 1993
15	<i>IGS2</i> region	Fan <i>et al.</i> , 1995

agar and grown at 30°C for 20 h. Four loopfuls of cells were carefully scraped from the agar and suspended in 1 ml TEME buffer (100 mM Tris, pH 8.0, 5 mM EDTA, 0.5% β -mercaptoethanol). PFGE was performed on a CHEF II gel apparatus (Bio-Rad). Plugs were loaded onto a 1% agarose gel in 0.5 \times TBE buffer, and the chromosomes were separated under the following conditions: block 1, 250–900 switch at 3 V/cm at 14°C for 46 h; block 2, 170–300 switch at 4 V/cm at 14°C for 48 h. The gel was then stained in ethidium bromide (0.5 μ g/ml) for 15 min, destained in distilled water for 15 min, and photographed using a Polaroid system.

Hybridization

For hybridization experiments, gels were blotted under neutralizing (20 \times SSC) conditions using capillary transfer. Briefly, the gel was submerged in 0.25 M HCl for 10 min, rinsed in distilled water, and submerged in denaturation solution (0.5 N NaOH, 1.5 M NaCl) for 30 min at room temperature (RT). The gel was briefly washed in distilled water and then submerged in neutralizing solution (0.5 M Tris/HCl [pH 7.5], 3 M NaCl) for 30 min at RT. Finally, the gel was rinsed in distilled water and blotted as described elsewhere (Maniatis *et al.*, 1989). After blotting, the membrane was cross-linked under UV light, rinsed in distilled water, and air dried for 30 min. The membrane was then wrapped in aluminum foil until further use.

To determine the corresponding chromosome for each generated linkage group, markers from each linkage group were labeled and hybridized to chromosomal blots of the two parental strains using a digoxigenin (DIG) labeling and detection system (Roché Pharmaceuticals, Nutley, NJ) according to the manufacturer's instructions.

To develop probes from AFLP and RAPD markers, PCRs were carried out (as described above), and the PCR products were separated on gels of 1.5% agarose in 1 \times TAE for 5 h at 3.5 V/cm. Bands of interest were then excised from the gel and purified with a QIAquick gel extraction kit (Qiagen). DIG high prime labeling reactions were carried out using 300 ng–1 μ g of DNA per marker following the manufacturer's instructions (Roché Pharmaceuticals). Probes were stored at –20°C until further use. To obtain DNA coding markers, PCRs were performed, and the markers were directly purified from the PCR product using a QIAquick PCR purification kit (Qiagen). DIG hybridization and detection was carried out following the manufacturer's instructions (Roché).

RESULTS

Marker Development

AFLP markers. Of 25 primer pairs initially tested using the two parental strains, 10 were selected for subsequent analysis (Table 4). For each primer pair, an average of 166 AFLP fragments were obtained. The number of polymorphisms distinguished between the parents ranged from 5 (for primer pair MgaEca) to 22 (for primer pair MgaEgtc), with an average of 14.1 polymorphic fragments (markers) per primer pair (see also Table 4). Of the polymorphic AFLP markers, 63 markers were present only in parent A (B3502), and 78 markers were found only in parent B (B3501). Of these 141 AFLP markers, 31 showed significant segregation distortion, and 16 expressed highly distorted segregation ratios (χ^2 values > 7.88; P < 0.005). AFLP bands are quite numerous and very close to each other. All markers were scored three times and ambiguous markers were discarded.

RAPD markers and genes as markers. RAPD markers were selected for analysis if they segregated among the parental strains and were well isolated and easy to purify from agarose gels. Consequently, only 6 primer combinations of an initial 64 tested combinations and 4 of 10 single primers tested satisfied these criteria (Table 4). A total of 31 markers was obtained: 13 markers were present only in parent A and 18 markers were present only in parent B (Table 4). Two RAPD markers showed segregation distortion (χ^2 values < 3.84; P > 0.05).

Gene-coding DNA sequence markers. Of 15 surveyed genes, 11 were screened by sequencing, and the 4 *Ste* genes were tested for the presence and the absence of a PCR fragment. Of the 11 genes that were partially sequenced from the parental strains, only 3 genes (*CAP64*, *URA5*, *CnLAC1*) displayed polymorphisms between the parents (Table 4). For these 3 genes, a total of five codominant single nucleotide polymorphisms (SNPs) was obtained with one SNP each for *CAP64* and *CnLAC1* and three SNPs for *URA5*. From the 4 *Ste* genes, four markers were scored for the presence or absence of a single amplicon. Consequently, a total of nine markers was obtained with significant balanced segregation ratios. The mating type of each progeny was determined by backcrosses with each parent and other tester strains.

Combined markers. For linkage analysis, segregation data from the progeny were combined for all markers, and the linkage analysis was based on a total of 181 dominant/recessive markers. Seventy-seven markers (bands) were present only in parent A, 99 markers (bands) were detected

TABLE 4

Results of the Development of AFLP Markers, RAPD Markers, and Gene-Encoding DNA Markers

Marker	Total AFLP fragments	Total number of polymorphic fragments			Skewed markers*	Unlinked markers		
		Total	Parent A	Parent B				
AFLP	MgtEgt	144	13	6	7	3	0	
	MtgEtg	134	15	8	7	5	1	
	MgaEca	204	12	9	3	2	3	
	MgtEca	150	5	3	2	0	1	
	McaEgt	142	13	6	7	2	2	
	MacEgt	132	12	4	8	3	0	
	McaEtg	194	13	5	8	1	1	
	MacEgtc	163	19	5	14	4	3	
	MgaEgtc	240	22	7	15	5	2	
	MtgcEtg	155	17	10	7	6	5	
RAPD	A1B11	—	2	1	1	0	2	
	A2B10	—	1	0	1	0	1	
	A7A8	—	2	0	2	0	0	
	A7A9	—	2	0	2	0	0	
	A7A10	—	4	1	3	0	1	
	A7A11	—	4	2	2	0	1	
	A1	—	3	3	0	1	1	
	A2	—	4	1	3	0	1	
	A17	—	4	3	1	1	2	
	B15	—	5	2	3	0	0	
	Gene-encoding DNA regions	<i>Ste11</i>	—	1	0	1	0	0
		<i>Ste12</i>	—	1	0	1	0	0
		<i>Ste20a</i>	—	1	1	0	0	0
		<i>Ste20α</i>	—	1	0	1	0	0
		<i>CAP64¹</i>	—	1	0	1	0	0
<i>URA5¹</i>		—	3	3	0	0	0	
<i>CnLAC1¹</i>		—	1	1	0	0	0	
			Total: 181	Total A: 81 (44.7%)	Total B: 100 (55.3%)	Total: 33 (18%)	Total: 27 (15%)	

Note. AFLP markers are listed based on the different primer combinations, followed by the number of obtained AFLP fragments per primer pair and the number of polymorphic fragments (markers); the markers are divided according to their parental origin. The number of skewed and the number of unlinked markers are given for each primer combination. The same scheme was applied for the RAPD markers and the coding DNA markers. The total of markers for each specified column is given at the bottom of the table.

* Markers were called skewed if they exceeded a χ^2 value of 3.84 with $P > 0.05$ (markers did not segregate significantly at a 50:50 ratio).

¹ Codominant markers.

only in parent B, and 5 codominant markers were obtained from sequence data (see Table 4). From these 181 markers, 148 segregated in a ratio of 1:1, the segregation of 15 markers was slightly skewed ($3.84 < \chi^2 < 10.81$), and 18 markers exhibited highly distorted segregation ($\chi^2 > 10.81$; $P < 0.001$). The proportion of markers with skewed segregation ratios was higher for markers inherited from parent B (21 of 33 markers) than for markers inherited from parent A (12 of 33 markers).

Linkage Analysis

To determine the optimal parameters to generate the linkage map, several combinations of LOD thresholds

(3.0, 4.0, and 5.0) and recombination fraction values (0.20–0.30) were applied to the data set. With recombination values less than 0.24, the linkage groups (LG) obtained with these data were more stable. Changing the LOD values from 3.0 to 5.0 did not seem to influence the results. Based on these findings, a LOD threshold of 5.0 and recombination fractions varying from 0.20 to 0.24 between two adjacent markers were used to generate the main framework of the linkage map. Only 148 markers with normal segregation ratios ($\chi^2 < 3.84$; $P > 0.05$) were used. Fourteen linkage groups with at least 4 markers each were obtained, as well as several smaller linkage groups with 2 and 3 markers.

TABLE 5
Generated Linkage Groups for *Cryptococcus neoformans* var. *neoformans*, Serotype D

	Name of LG/CH	Number of markers	Size of LG (in cM)	Average spacing of markers
Major LGs/ Chromosomes (CH)	LG1	19	151.5	7.97
	CH2	9	125.6	13.95
	CH3	11	76.2	6.93
	CH4/5/6-A	9	124.9	13.9
	CH4/5/6-B	8	53.8	6.7
	CH4/5/6-C	6	43	8.6
	CH7	8	77.6	9.7
	LG8	17	117.9	6.9
	LG9	12	112.6	9.4
	LG10	7	94.5	13.5
	LG11	7	57.8	8.2
	LG12	6	55.2	9.2
	LG13	4	28.8	9.6
	LG14	4	3.2	0.8
Small LGs (SLG)	SLG1	2	0	0
	SLG2	2	1.3	0.65
	SLG3	2	22.3	11.1
	SLG4	2	7.8	8.4
	SLG5	2	40.8	20.4
	SLG6	3	27	9
	SLG7	3	9.7	3.2
	SLG8	3	13.6	4.5
Distorted LGs (DLG)	DLG1	4	38.7	9.7
	DLG2	5	52.5	10.5

Note. Linkage groups (LG) are divided into main LGs, small LGs, and distorted LGs. The columns indicate the number of markers for each LG, the size of each LG, and the average spacing of the markers within each LG.

To include markers with slightly skewed segregation ratios and to map markers that were unlinked, the recombination fraction values were relaxed in a stepwise fashion, while the LOD was maintained at 5.0, and one marker was added at a time to the stable linkage groups. Markers that significantly changed the order of LGs or the composition of the map were not included.

The final linkage map (based on >3 markers) was constructed from 127 of 181 markers, consisting of 14 major LGs (Fig. 2). The map covers a total length of 1142.6 cM with an average distance of 8.9 cM between markers. The largest LG has 19 markers and is 151.5 cM in size, and the smallest LG contained 4 markers and is 3.1 cM in size (Table 5). Eight small LGs, containing 2 and 3 markers, were also obtained; they vary in size from 0 to 40.8 cM. Twenty-six markers could not be assigned to any linkage group. Nine of the markers with highly skewed segregation ratios formed 2 LGs with 4 and 5 markers (Table 5). One of these 2 LGs contained only markers that were skewed toward parent A, and the other LG contained markers that were skewed toward parent B. To

visualize the skewness of markers within linkage groups, a chart was generated. For 100 progeny, the recombination frequency of each marker was calculated and plotted against the distal location (in cM) of the marker on the linkage group (Microsoft Excel, version 5.0). This calculation was applied to each major LG and to the 2 skewed LGs individually, and all LGs were combined in the same chart (Fig. 3). A recombination frequency of 0.5 denotes independent segregation. Figure 3 shows that all major LGs are located slightly below the level of 0.5 recombination frequency, indicating that they are skewed toward parent B. The 2 highly skewed LGs are graphed as expected. The LG with markers skewed toward parent A was located near the highest recombination fraction of 1.0, and the LG with markers skewed toward parent B was positioned near the lowest level of the recombination fraction (zero) (Fig. 3).

If small LGs and skewed LGs are included in the linkage map, the genome size expands to a total length of 1356.3 cM. However, using a combination of different methods to calculate the genome sizes (Chakravarti *et al.*, 1991; Hulbert *et al.*, 1988; Remington *et al.*, 1999), our data suggest a total length of 1917 cM for the genetic linkage map of *C. neoformans*. Based on this estimation, the linkage map generated in this study spans about 71% of the total length of the genome of *C. neoformans* var. *neoformans*.

Linkage Plot Analysis

To further examine associations among loci including possible higher-level (e.g., two-way) interactions, linkage values among loci were plotted to facilitate visualization of LGs. First, segregating markers were ordered by LG and by their map order within LGs. A matrix of recombination fractions (Rf) was then calculated for all pairs of loci and plotted as a shaded graph with lower recombination values shaded highest. Distinct LGs are readily viewed in the resulting linkage plot (Fig. 4) by their darker shading. Most LGs also are segmented into two major shaded portions separated by a lighter shading probably representing the centromere region for each chromosome. In addition, regions of darker shading suggest possible associations between several linkage groups, particularly between LG 9 and CH4/5/6-A and between CH2 and LG 12. Several unlinked loci can also be seen to have little or no linkage with the main 14 groups. This method of graphically imaging complex interlocus interactions is similar to the clustering methods proposed for analysis of gene expression used in genome analysis (e.g., Eisen *et al.*, 1998), except that in this case the data are estimating linkage instead of gene expression. Computer programs for calculating and plotting the Rf matrix are available upon request from the authors.

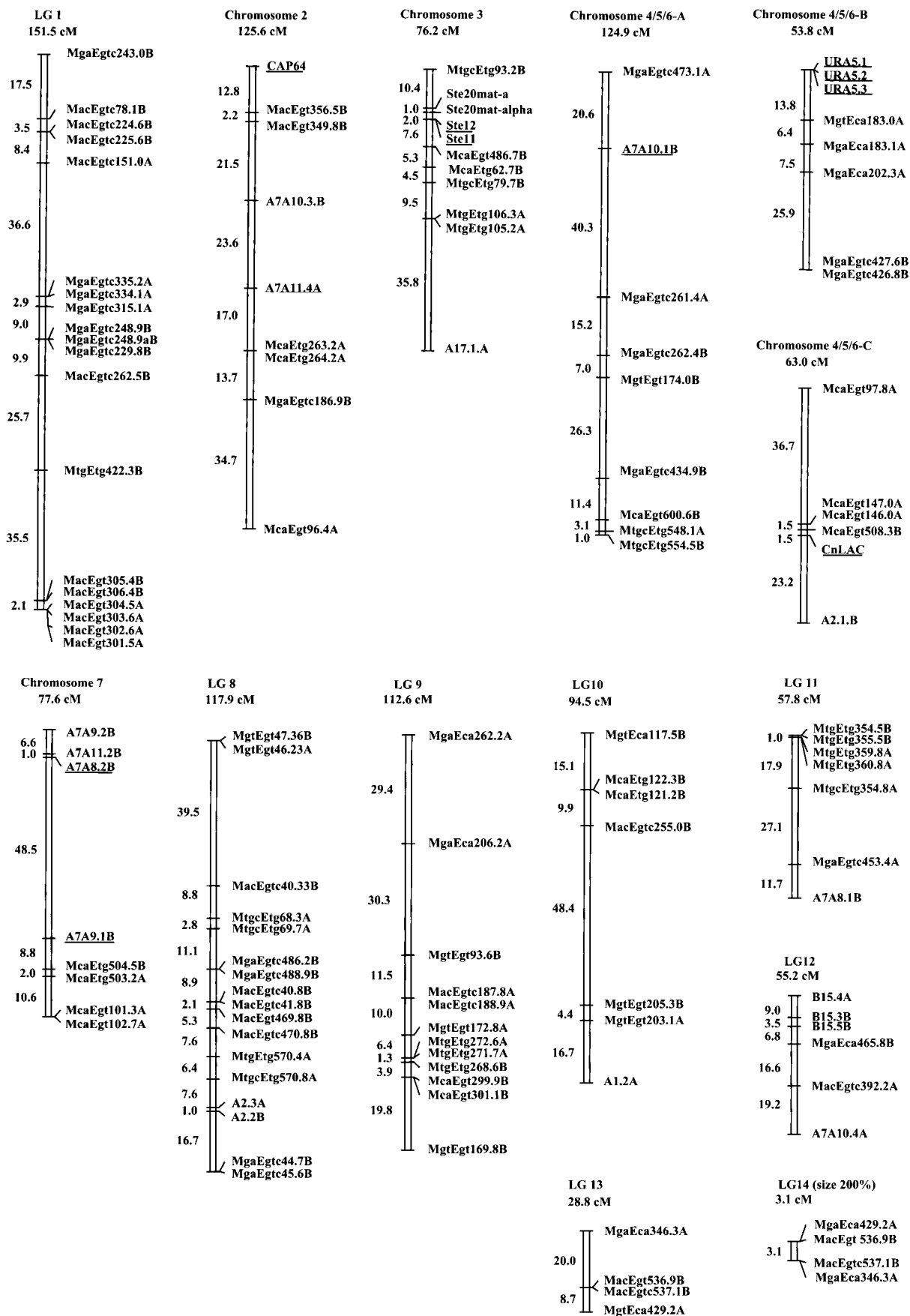


FIG. 2. Genetic linkage map of *Cryptococcus neoformans* var. *neoformans* based on 127 molecular markers (AFLPs, RAPDs, and gene-encoding sequences) at a LOD score of 5.0. Markers that are underlined confirmed the chromosome location of these linkage groups.

3

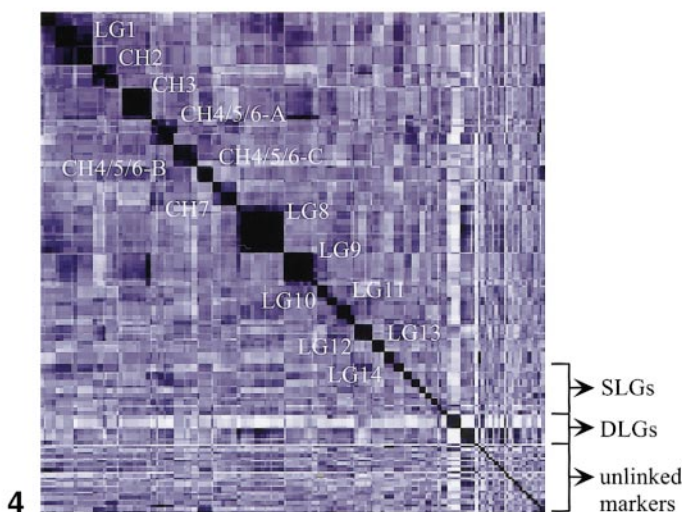
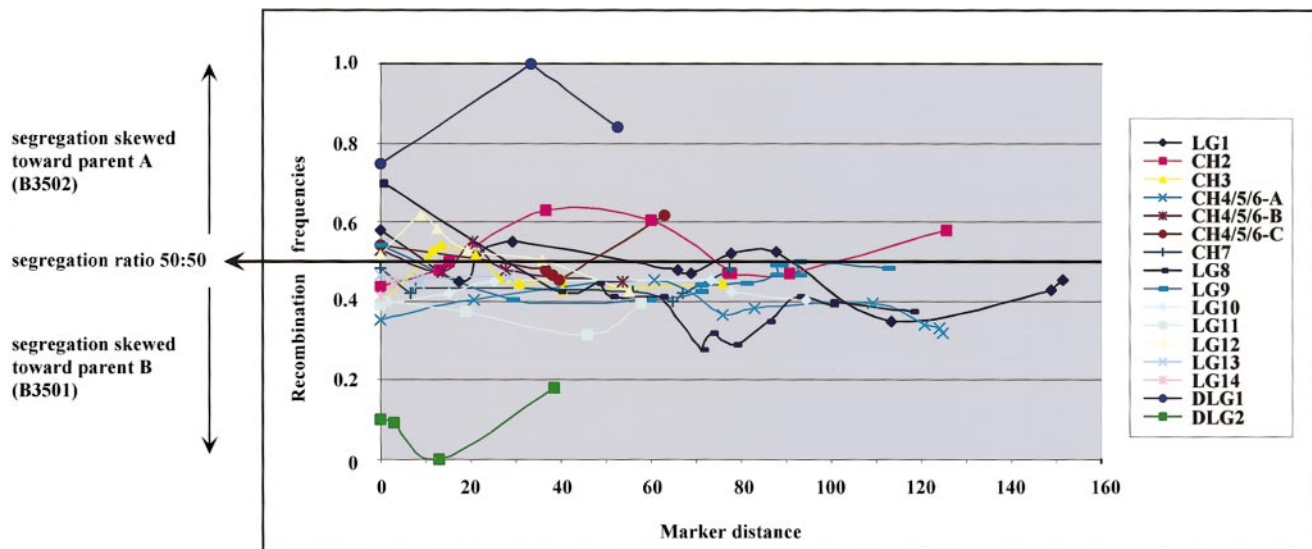


FIG. 3. Segregation plot for major and skewed linkage groups (LG). The y axis represents the recombination frequency values; the x axis gives the distance between the markers on the linkage group. Each curve demonstrates a linkage group, a distorted LG (DLG), or a chromosome (CH); see right-hand legend for identification and color code. The black horizontal arrow indicates the segregation ratio of 50:50, and the two vertical arrows on the left indicate the direction of skewness toward parent A and parent B.

FIG. 4. Linkage plot based on recombination fraction of pair wise comparisons of all 181 markers, ordered by linkage groups/chromosomes. Color coding: white, no linkage; black-blue, strongest linkage. SLGs, small linkage groups; DLGs, distorted linkage groups.

The AFLP markers appear evenly distributed throughout the linkage map. No LGs consist of markers derived from only one parent. The majority of RAPD markers map to a cluster within LG 7. Most of the skewed markers that were integrated into the map are located at the ends of LGs. For several LGs, these regions are also characterized by none or only few recombinational events, and these might represent telomeric regions. Some regions within LGs, such as in LGs 8 and 10, have fewer markers. The largest such gap is located on LG 7 and spans 48.5 cM. No significant marker clustering

was observed. Instead, there were many small groups of tightly linked clusters of two to four markers. Predictably, these groups of tightly linked markers exhibited no recombination (genetic distance was zero).

Crossover Events

The amount of recombination between markers within and among LGs varied from 0 to 31, with the highest numbers found for LG 7. The average number of cross-

over events was 6.9 per LG. As expected, the number of crossovers increased with higher distance between markers (data not shown). Some of the higher values, which were detected in several LGs, may reflect recombinational hot spots throughout the genome. However, since the probability of crossovers increases with the distance between two markers, the higher crossover values may be due to large distances between two markers within LGs rather than hot spots. More markers per LG would likely decrease the observed recombination between markers and within LGs.

Mating Locus

The mating loci for *C. neoformans* var. *neoformans* have been located on chromosome 3 (Fig. 2) (Wickes *et al.*, 1994). Four *Ste* genes, *Ste11- α* , *Ste12- α* , *Ste20-a*, and *Ste20- α* , have been mapped to this chromosome. Based on our linkage analysis, all four genes are tightly linked with no and little genetic distance separating them (Fig. 3). The order of these genes seems to be *Ste11*, *Ste12*, *Ste20- α* , and *Ste20-a*. The MAT α and MATa loci map next to each other on the chromosome and are separated by only 1 cM. Chromosome 3 has a physical size of 1.88 Mb and spans a genetic distance of 76.2 cM. Since the entire mating locus has a genetic distance of 3 cM, this locus spans approximately 74 kb.

PFGE Analysis and Assignment of LGs to Their Corresponding Chromosomes

The karyotypes of the parental strains were obtained by PFGE, as shown in Fig. 5. The size of each chromosome was estimated based on visual examination using the chromosomes of *Saccharomyces cerevisiae* and *Hansenula wingei* as size standards. This analysis revealed 10 clearly visible chromosomal bands for parent B (B3501) and 11 for parent A (B3502). Based on the greater intensity of bands 4 and 7 of parent B and bands 6 and 11 of parent A, 13 chromosomes are proposed for both strains. For both parental strains, the largest chromosome has a physical size of 2.44 Mb (CH 1), and the smallest chromosome is about 806 kb (CH 13). The calculated sizes of chromosomes 5 and 9 are also identical for both parents; the size variations between parental chromosomes 2, 3 and 8 are slight. All the other chromosomes differ in their sizes, indicating the presence of chromosomal length polymorphisms (CLPs). To assign the generated LGs to their appropriate chromosomes, selected markers (preferably

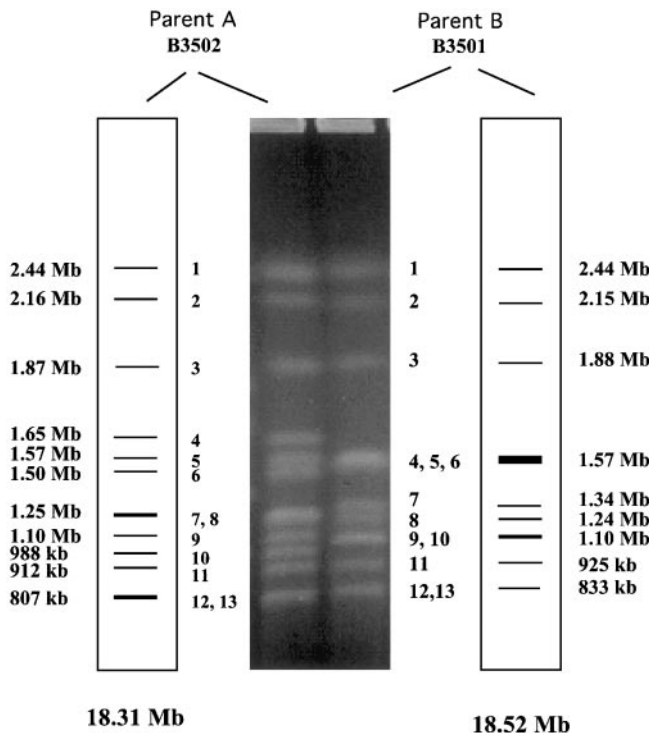


FIG. 5. Karyotypes of the parental strains obtained by PFGE analysis and diagrams of the karyotypes with sizes and nomenclature of the chromosomes.

RAPDs and gene-encoding DNA markers) were hybridized to PFGE blots of the two parental strains. To date, six LGs have been assigned that correspond to chromosomes 2 to 7 (Fig. 2, Table 5). The chromosomal location of these LGs was confirmed by one or more markers (underlined in Fig. 2).

The estimate of the total genome size for both parents varies between 18.31 Mb for parent A and 18.52 Mb for parent B (Fig. 5). Since the total length of this linkage map is 1356.3 cM, 1 cM would correspond to approximately 13.6 kb. However, this value is not consistent with the calculated sum of the physical size and length in cM of the individual chromosomes. There is no linear correlation between the physical size of a chromosome and its genetic length across the genome. For example, CH 3 has a physical size of 1.88 Mb and a genetic length of 76.2 cM, yielding 24.7 kb/cM, and CH 7 is approximately 1.29 Mb (average sizes for both parents with a genetic length of 77.6 cM), resulting in 16.6 kb/cM. Since this map covers about 71% of the total length of the genome, these values will most likely change, as the map becomes more complete.

DISCUSSION

This moderately dense genetic linkage map is the first to be developed for *C. neoformans*, and it covers 71% of the total genome. To generate a linkage map, one needs a mapping population with large numbers of progeny and easily readable markers. The markers used in this study included AFLP markers, RAPD markers, and gene-encoding DNA sequences. Most were AFLP markers, which have been used increasingly to develop genetic linkage maps, especially for plants (Lu *et al.*, 1998; von Heusden *et al.*, 2000) and phytopathogenic fungi (Van der Lee *et al.*, 1997). AFLP markers are advantageous because a large number of markers can be rapidly generated to provide high-resolution mapping (Jones *et al.*, 1997). AFLP markers are also more reproducible than RAPD markers. AFLPs span the genome and are sensitive enough to detect SNPs. The initial phase in the development of AFLP markers includes the total digestion of the genome with *Eco*RI and *Mse*I, which is expensive, compared to methods such as RAPDs. However, once the preamplification reactions are obtained for each progeny strain, more than 100 final reactions can be carried out with different primer pairs per sample. Because of these advantages, we chose to use the AFLP method to develop the majority of our markers.

One hundred eighty-one markers were analyzed to generate the linkage map. Screening 10 primer pairs, 141 AFLP markers were obtained. In comparison with RAPDs, 64 combinations of 10-mer arbitrary primers and 10 single 10-mer oligonucleotides were tested, but only 31 RAPD markers were found that met our criteria. An even smaller number of polymorphisms was observed when gene-encoding sequences were compared between the parental strains. Eleven different genes were screened, but only five SNPs were detected among the parents.

One reason for the low level of polymorphisms might be that the parental strains, B3501 and B3502, which were used in the cross to obtain sexual progeny, are sibling strains. The fact that their karyotypes revealed chromosomal length polymorphisms, suggesting certain levels of genomic differences between them, is not reflected by the markers, which characterize only small polymorphisms, such as single nucleotide polymorphisms. CLPs have been studied in a variety of other fungi, including *Candida albicans* (Thrash-Bingham and Gorman, 1992), *Coprinus cinereus* (Zolan *et al.*, 1994), *S. cerevisiae* (Camasses, 1996), and *Yarrowia lipolytica* (Casaregola *et al.*, 1997), and can have different origins (Zolan, 1995). A future

study of the nature of CLPs would certainly enrich the knowledge about the genome of *C. neoformans*.

One hundred progeny strains from this cross were used in this analysis. The size of the progeny population is important for the development of a genetic linkage map, because a sufficient number of recombinational events are needed to calculate an accurate linkage of the markers. The number of progeny used in this study is similar or larger than progeny sizes used to generate genetic linkage maps for other organisms, such as plants, plant pathogenic fungi, and cultivated fungi. For example, to create a genetic linkage map for the Oomycete *Phytophthora infestans*, 73 single spore progenies were analyzed, yielding 191 markers (Van der Lee *et al.*, 1997), and a genetic linkage map was produced for *Agaricus bisporus* with 52 haploid offspring and 64 markers (Kerrigan *et al.*, 1993). Other linkage studies in plants used numbers of progeny that varied between 35 (Su *et al.*, 1999) and 106 (Lespinasse *et al.*, 2000).

To determine linkage relationships between markers, the progeny strains were first screened for all markers that were polymorphic between the parents. The segregation ratios were calculated, and the significance of these values was tested by applying a simple goodness of fit test (χ^2 test). Of 181 markers, 33 (18%) showed segregation ratios that were skewed toward either parent A (12 of 33; 36.4%) or parent B (21 of 33; 63.6%). Clearly, most skewed markers were inherited from parent B. This phenomenon has been noted by others; there are several reports in which the distortion of marker segregation was higher for the markers of one parent (Debener and Mattiesch, 1999; Su *et al.*, 1999; von Heusden *et al.*, 2000). Skewed markers may reflect any of several genomic processes. They have been associated with distortion factors, such as self-incompatibility alleles, or with the expression of linked lethal genes (Bert *et al.*, 1999; Lu *et al.*, 1998). Skewed markers might also indicate epistatic effects of genes that suppress the expression of linked genes. Other explanations for segregation distortion can be attributed to sampling errors, such as incorrectly scoring bands or an inadequate number of progeny (Lu *et al.*, 1998). Markers with distorted segregation ratios were also observed when the mapping population was obtained from parents with different genome sizes (Bert *et al.*, 1999). However, these latter explanations are unlikely to pertain in this study because the population size of 100 progeny was sufficiently large, and all markers were scored repeatedly to ensure accuracy. Skewed markers in this analysis are more likely caused by genetic interactions throughout the genome as well as other distortion factors.

Initial linkage analyses were carried out using only markers with normal Mendelian segregation ratios, and markers with segregation distortion were added later to the established linkage map. The MAPMAKER program was used to analyze 155 markers and generated 14 major linkage groups, 8 smaller linkage groups, and 2 linkage groups with exclusively skewed markers, spanning a cumulative size of 1356.3 cM. Based on calculations proposed by Hulbert and coworkers (1988), the estimated total genome size of *C. neoformans* is approximately 1917 cM. With the limited number of markers, it is not surprising that the coverage is only 71%. Highly saturated genetic linkage maps of other organisms have been published, consisting of 708 markers (Lespinasse *et al.*, 2000), 508 markers (Remington *et al.*, 1999), and 463 markers (Bert *et al.*, 1999). Therefore, many more markers are necessary to saturate the genetic linkage map of *C. neoformans*, and this work is in progress.

No large groups of markers were densely clustered on the linkage map of *C. neoformans*. In other linkage studies, this phenomenon was observed commonly, and two explanations have been suggested. First, marker clusters represent regions where recombination is suppressed, perhaps corresponding to centromere and/or telomere regions (Grattapaglia and Sederoff, 1994; Lespinasse *et al.*, 2000). Second, the clustering of markers has been associated with the AFLP markers. The AFLP method is more sensitive than the RFLP method for the detection of SNPs, which often occur in repetitive sequences near the centromere and/or telomere. Therefore, since AFLP markers are more likely to detect SNPs, they may produce clustered markers (Qi *et al.*, 1998). This cluster phenomenon of AFLP markers was associated with more dense linkage maps. Hence, additional AFLP markers may reveal clustering in this linkage map.

There is still no explanation for the two linkage groups that consist of exclusively skewed markers. One group is composed of markers inherited only from parent A, and the other group includes only markers from parent B. This result is reminiscent of sex chromosomes. It is also possible that these linkage groups are "pseudo" linkage groups rather than real linkage groups. In reality, these markers might be dispersed throughout the genome, but they could be linked because of strong epistatic effects. The reason that other skewed markers fit well within linkage groups could be that the segregation ratios of these markers are less skewed. To explore these possibilities, we will isolate key markers from the pseudo linkage groups and hybridize these markers to a PFGE blot to determine their actual location and linkage status.

Karyotyping was performed in this study to confirm the number of chromosomes of both parental strains (B3501 and B3502) and to calculate the physical size of the genome. Both parents revealed 13 chromosomes. Two chromosome standards were used, and seven different PFGE gels were analyzed to provide a correct size for each of the 13 chromosomes. An approximate size of 18.53 and 18.31 Mb was calculated for the parental strains B3501 and B3502, respectively. In recent years, several karyotyping studies have been performed on these strains. The number of chromosomes that we observed is consistent with data from other laboratories (Wickes *et al.*, 1994; Perfect *et al.*, 1989). However, there are discrepancies in the chromosomal sizes, especially in the range of larger chromosomes. In this study, the largest chromosome was determined to be 2.44 Mb, similar to the finding of Boekhout *et al.* (1997). However, Wickes *et al.* (1994) estimated a size of 3.87 Mb. Consequently, their estimated average genome size of *C. neoformans* var. *neoformans* of 21.6 Mb was larger than that in our analysis (Wickes *et al.*, 1994). It is difficult to calculate chromosomal sizes because of the influence of a number of PFGE running parameters, including the gel concentration, switch times, running times, and voltage. To minimize the effects of these conditions, we analyzed many gels and used two different chromosome standards to achieve a physical genomic size of approximately 18.4 Mb.

To determine the chromosomal locations of the generated LGs, blots of parental chromosomes were hybridized with markers from each LG. This approach identified the chromosomes on which six LGs were located. Chromosomes 2, 3, and 7 were clearly identified, but we have not resolved the specific chromosomal locations of the three linkage groups that hybridized to chromosomes 4, 5, and 6. However, chromosomes 4, 5, and 6 have identical migration rates in the gel, and therefore it was not possible to determine the exact location of each chromosome. Improved separation of these chromosomes and additional hybridizations will be necessary to assign the remaining linkage groups to their appropriate chromosomes.

CONCLUSIONS

This investigation produced a genetic linkage map of *C. neoformans*, including markers, mapping data, and information on recombination parameters. This information will support laboratory studies on the genetics and pathogenicity of *C. neoformans*. This map provides the first overview of this genome and a basis for determining the

organization of the genome and genomic rearrangements, such as chromosomal length polymorphisms.

With the data on recombination frequencies, crossover events, and genetic linkage, the general meiotic behavior of *C. neoformans* could be investigated and compared with other fungal or nonfungal species. Furthermore, by comparing the genetic linkage map with the physical map, potential recombination hotspot and centromeres could be detected.

An important application of a linkage map is to tag genes. If a gene of interest is closely linked to a marker on the map, it is likely that both are inherited together from parents to their offspring. This marker can then be used to determine the genomic location of the linked gene in subsequent genetic studies. By knowing the position of the marker in relation to the gene of interest, one can identify the fragments and sequences of the gene by chromosome walking. This approach permits one to localize genes that control simple or complex traits (e.g., virulence, drug resistance), which are important areas of research in *C. neoformans*.

Furthermore, this map may provide a framework for the recently initiated genome sequencing project. It will help to assign sequence contigs to chromosomes and guide their order and orientation. This is especially important for regions with repetitive sequences, where errors may occur with the assembling of contigs. With the availability of a genetic linkage map, knowledge of the karyotype, and the forthcoming genomic sequence of *C. neoformans* var. *neoformans*, we can start working toward a thorough understanding of the genome of this emerging human pathogen.

ACKNOWLEDGMENTS

We thank Timothy James and John Mercer for providing the C++ program for calculating the Rf matrix and for the program to plot the Rf matrix, respectively. This research was supported by Public Health Service Grants AI25783 and AI44975 from the National Institute of Health. This is a publication of the Duke University Mycology Research Unit.

REFERENCES

- Bert, P. F., Gharmet, G., Sourdille, P., Hayward, M. D., and Balfourier, F. 1999. A high-density molecular map for rye grass (*Lolium perenne*) using AFLP markers. *Theor. Appl. Genet.* **99**: 445–452.
- Boekhout, T., and van Belkum, A. 1997. Variability of karyotypes and RAPD types in genetically related strains of *Cryptococcus neoformans*. *Curr. Genet.* **32**: 203–208.
- Boekhout, T., van Belkum, A., Leenders, A. C. A. P., Verbrugh, H. A., Mukamurangwa, P., Swinne, D., and Scheffers, W. A. 1997. Molecular typing of *Cryptococcus neoformans*: Taxonomic and epidemiological aspects. *Int. J. Syst. Bacteriol.* **47**: 432–442.
- Camasses, A. 1996. Natural translocation of a large segment of chromosome III to chromosome I in a laboratory strain of *Saccharomyces cerevisiae*. *Curr. Genet.* **30**: 218–223.
- Casadevall, A., and Perfect, J. R. 1999. *Cryptococcus neoformans*. ASM Press, Washington, DC.
- Casaregola, S., Feynerol, C., Diez, M., Fournier, P., and Gaillardin, C. 1997. Genomic organization of the yeast *Yarrowia lipolytica*. *Chromosoma* **106**: 380–390.
- Chakravarti, A., Lasher, L. K., and Reefer, J. E. 1991. A maximum likelihood method for estimating genome length using genetic linkage data. *Genetics* **128**: 175–182.
- Debener, T., and Mattiesch, L. 1999. Construction of a genetic linkage map for roses using RAPD and AFLP markers. *Theor. Appl. Genet.* **99**: 891–899.
- Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* **95**: 14863–14868.
- Franzot, S. P., Salkin, I. F., and Casadevall, A. 1999. *Cryptococcus neoformans* var. *grubii*: Separate varietal status for *Cryptococcus neoformans* serotype A isolates. *J. Clin. Microbiol.* **37**: 838–840.
- Grattapaglia, D., and Sederoff, R. 1994. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: Mapping strategy and RAPD markers. *Genetics* **137**: 1121–1137.
- Heitman, J., Allen, B., Alspaugh, J. A., and Kwon-Chung, K. J. 1999. On the origins of congenic *MAT α* and *MATa* strains of the pathogenic yeast *Cryptococcus neoformans*. *Fungal Genet. Biol.* **28**: 1–5.
- Hulbert, S. H., Ilott, T. W., Legg, E. J., Lincoln, S. E., Lander, E. S., and Michelmore, R. W. 1988. Genetic analysis of the fungus, *Bremia lactucae*, using restriction fragment polymorphisms. *Genetics* **120**: 947–958.
- Jones, N., Ougham, H., and Thomas, H. 1997. Markers and mapping: We are all geneticists now. *New Phytol.* **137**: 165–177.
- Kerrigan, R. W., Royer, J. C., Baller, L. M., Kohli, Y., Horgen, P. A., and Anderson, J. B. 1993. Meiotic behavior and linkage relationships in the secondarily homothallic fungus *Agaricus bisporus*. *Genetics* **133**: 225–236.
- Kwon-Chung, K. J. 1976. Morphogenesis of *Filobasidiella neoformans*, the sexual state of *Cryptococcus neoformans*. *Mycologia* **68**: 821–833.
- Kwon-Chung, K. J., and Rhodes, J. C. 1986. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. *Infect. Immun.* **51**: 218–223.
- Lander, E. S., and Green, P. 1987. Construction of multilocus genetic linkage maps in humans. *Proc. Natl. Acad. Sci. USA* **84**: 2363–2367.
- Lander, E. S., Green, P., Abrahamson, A., Barlow, A., and Daly, M. J. 1987. MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* **1**: 174–181.
- Lengeler, K. B., Wang, P., Cox, G. M., Perfect, J. R., and Heitman, J. 2000. Identification of the *MATa* mating type locus of *Cryptococcus neoformans* reveals a serotype A *MATa* strain thought to have been extinct. *Proc. Natl. Acad. Sci. USA* **97**: 14455–14460.
- Lespinasse, D., Rodier-Goud, M., Grivet, L., Leconte, A., and Legnate, H. 2000. A saturated genetic linkage map of rubber tree (*Hevea* spp.)

- based on RFLP, AFLP, microsatellite, and isozyme markers. *Theor. Appl. Genet.* **100**: 127–138.
- Lu, Z.-X., Sosinski, B., Reighard, G. L., Baird, W. V., and Abbott, A. G. 1998. Construction of a genetic linkage map and identification of AFLP markers for resistance to root-knot nematodes in peach rootstocks. *Genome* **41**: 199–207.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Morton, N. E. 1955. Sequential tests for the detection of linkage. *Am. J. Hum. Genet.* **7**: 277–318.
- Perfect, J. R., Magee, B. B., and Magee, P. T. 1989. Separation of chromosomes of *Cryptococcus neoformans* by pulsed field gel electrophoresis. *Infect. Immun.* **57**: 2624–2627.
- Polacheck, I., and Lebens, G. A. 1989. Electrophoretic karyotype of the pathogenic yeast *Cryptococcus neoformans*. *J. Gen. Microbiol.* **135**: 65–71.
- Qi, X., Stam, P., and Lindhout, P. 1998. Use of locus-specific AFLP markers to construct a high density molecular map in barley. *Theor. Appl. Genet.* **96**: 376–384.
- Remington, D. L., Whetten, R. W., Liu, B.-H., and O'Malley, D. M. 1999. Construction of an AFLP genetic linkage map with nearly complete genome coverage in *Pinus taeda*. *Theor. Appl. Genet.* **98**: 1279–1292.
- Sia, R. A., Lengeler, K. B., and Heitman, J. 2000. Diploid strains of the pathogenic basidiomycete *Cryptococcus neoformans* are thermally dimorphic. *Fungal Genet. Biol.* **29**: 153–163.
- Spitzer, S. G., and Spitzer, E. D. 1997. Isolation of *Cryptococcus neoformans* chromosome-specific probes using expressed sequence tags. *J. Med. Vet. Mycol.* **35**: 257–261.
- Still, C. N., and Jacobson, E. S. 1983. Recombinational mapping of capsule mutations in *Cryptococcus neoformans*. *J. Bacteriol.* **156**: 460–462.
- Su, X., Ferdig, M. T., Huang, Y., Huynh, C. Q., Liu, A., You, J., Wootton, J. C., and Wellem, T. E. 1999. A genetic map and recombination parameters of the human malaria parasite *Plasmodium falciparum*. *Science* **286**: 1351–1353.
- Thompson, J. R., Douglas, C. M., Li, W., Jue, C. K., Pramanik, B., Yuan, X., Rude, T. H., Toffaletti, D. L., Perfect, J. R., and Kurtz, M. B. 1999. A glucan synthase FKS1 homolog in *Cryptococcus neoformans* is single copy and encodes an essential function. *J. Bacteriol.* **181**: 444–453.
- Thrash-Bingham, C., and Gorman, J. A. 1992. DNA translocations contribute to chromosome length polymorphisms in *Candida albicans*. *Curr. Genet.* **22**: 93–100.
- Van der Lee, T., DeWitte, I., Drenth, A., Alfonso, C., and Govers, F. 1997. AFLP linkage map of the Oomycete *Phytophthora infestans*. *Fungal Genet. Biol.* **21**: 278–291.
- Von Heusden, A. W., Van Ooijen, J. W., Von Ginkel, R. V., Verbeek, W. H. J., and Wietsma, W. A. 2000. A genetic map of an interspecific cross in *Allium* based on amplified fragment length polymorphism (AFLP) markers. *Theor. Appl. Genet.* **100**: 118–126.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., van der Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**: 4407–4414.
- Wickes, B. L., Moore, T. D. E., and Kwon-Chung, K. J. 1994. Comparison of the electrophoretic karyotypes and chromosomal location of ten genes in the two varieties of *Cryptococcus neoformans*. *Microbiology* **140**: 543–550.
- Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531–6535.
- Wolfram, S. 1998. Mathematica 4. Vers. 4.0.1.0. Wolfram Research, Inc.
- Xu, J., Vilgalys, R., and Mitchell, T. G. 2000. Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. *Mol. Ecol.* **9**: 1471–1482.
- Zolan, M. E. 1995. Chromosome-length polymorphism in fungi. *Microbiol. Rev.* **59**: 686–698.
- Zolan, M. E., Heyler, N., and Stassen, N. 1994. Inheritance of chromosome-length polymorphisms in *Coprinus cinereus*. *Genetics* **137**: 87–94.