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Presence of α and **a** Mating Types in Environmental and Clinical Collections of *Cryptococcus neoformans* var. *gattii* Strains from Australia

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***Cryptococcus neoformans* var. *gattii* lives in association with certain species of eucalyptus trees and is a causative agent of cryptococcosis. It exists as two mating types, MATα and MATa, which is determined by a single-locus, two-allele system. In the closely related *C. neoformans* var. *neoformans*, the α mating type has been found to outnumber its a counterpart by at least 30:1, but there have been very limited data on the proportions of each mating type in *C. neoformans* var. *gattii*. In the present study, specific PCR primers were designed to amplify two separate α-mating-type genes from *C. neoformans* var. *gattii* strains. These were used to survey for the presence of the two mating types in clinical and environmental collections of *C. neoformans* var. *gattii* strains from Australia. Sixty-eight of 69 clinical isolates produced both α mating type-specific bands and were assumed to be of the α mating type. The majority of environmental isolates were also of the α mating type, but the a mating type was located in two separate areas. In one area, the a mating type outnumbered the α mating type by 27:2, but in the second area, the ratio of the two mating types was close to the 50:50 ratio expected for sexual recombination.**

Cryptococcus neoformans is an encapsulated, basidiomycetous yeast and is the causative agent of cryptococcosis, a rare but potentially serious disease of humans and animals (5). Two varieties of *C. neoformans* exist, and these differ biochemically, genetically, ecologically, and epidemiologically (13). *C. neoformans* var. *neoformans* has a worldwide distribution and has been associated with a variety of environmental sources, in particular, bird excreta (8) and decaying wood, forming hollows in a number of tree species (18). *C. neoformans* var. *gattii* has a more restricted global distribution, occurring in tropical and subtropical climates. Since 1989, *C. neoformans* var. *gattii* has been shown to have a specific ecological association with a number of eucalyptus species; *Eucalyptus camaldulensis* (river red gum), *Eucalyptus tereticornis* (forest red gum), *Eucalyptus rudis* (West Australian flooded gum), *Eucalyptus gomphocephala* (tuart) (4, 21, 22), *Eucalyptus grandis* (flooded gum), *Eucalyptus blakelyi* (Blakely's red gum), and *Angophora costata* (smooth-barked apple) (unpublished data). These trees are native to Australia, where a relatively high incidence of cryptococcosis due to *C. neoformans* var. *gattii* occurs in some native animals and indigenous human populations (6). They have also been exported to other tropical parts of the world, and *C. neoformans* var. *gattii* infections are also found in these regions (4). However, the role that the tree plays in the life cycle of this fungus and the nature of the infectious propagule are not well understood. Viable *C. neoformans* var. *gattii* cells have been found in the woody debris and detritus associated with the *Eucalyptus* species, and there appears to be a correlation between the flowering of the trees and the dispersal of the fungus (3). However, it is not known whether the fungus completes its life cycle on the tree to be shed as basidiospores or whether it propagates asexually and disperses as desiccated yeast cells. Basidiospores are favored as the infectious propagule, as they are small (<2 μm), can penetrate into the lung alveoli, and are more resistant to desiccation than yeast cells (13).

C. neoformans exists as two mating types, mating types α and **a**, with the mating type determined by a single locus with two idiomorphic alleles (10). In laboratory crosses, equal numbers of offspring of the **a** and α mating types are produced (10). However, surveys of *C. neoformans* var. *neoformans* isolates from clinical and environmental sources have shown that the α mating type outnumbers its **a** counterpart by ratios of 30:1 and 40:1, respectively (12, 19). In addition, population genetic studies of *C. neoformans* var. *neoformans* have indicated a clonal structure which may either influence or be influenced by this imbalance of mating types (7). The limited data available for *C. neoformans* var. *gattii* isolates found 84% of the clinical isolates to be of the α mating type, but no data are available for the mating type frequencies of *C. neoformans* var. *gattii* isolates in the environment (19).

C. neoformans var. *neoformans* strains of the α mating type have been associated with increased virulence in mice, which has prompted studies into the mating type locus (MATα) (15). Molecular studies have estimated MATα to be at least 75 kb in size (30). Within this locus, two α-mating-type genes have been identified: *MF*α and *STE12*α. The *MF*α gene contains a 114-bp open reading frame that encodes a pheromone precursor and that has homology to other fungal mating factors (20). *STE12*α is a homologue of the *Saccharomyces cerevisiae STE12* gene and is predicted to encode an 855-amino-acid protein. Between amino acids 85 to 201 lies a homeodomain with a high degree of identity to the *STE12* homeodomains of other fungal species (30). All molecular work on the mating-type locus of *C. neoformans* done to date has used *C. neoformans* var. *neoformans*. However, hybridization studies with *C. neoformans* var.

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TABLE 1. Environmental isolates of *C. neoformans* var. *gattii*

Isolate no.; source	Geographical location	Tree type	Isolate source	Date (mo/yr) isolated
Bal 1 to Bal 30; tree B1[a]	Balranald, NSW	*E. camaldulensis*	Woody debris	2/1996
401 Bal 2, 402 Bal 6, 402/1, 403 Bal 8, 405 Bal 2c, 406 Bal 2d, 407 Bal 2f, 408 B1, 409 B2, 410 B5[a]	Balranald, NSW	*E. camaldulensis*	Bark, woody debris, soil	12/1989 to 1/1990
404 H22b[a]	Hay, NSW	*E. camaldulensis*	Soil, wood nuts	12/1989 to 1/1990
Ad 1 to Ad 26, tree A1[a]	Adelaide, SA	*E. camaldulensis*	Woody debris	1/1996
GC 1 to GC8, GC 10 to GC 12, GC 15 to GC 18, GC 21 to GC 30; tree GC1[a]	Gold Coast, QLD	*E. tereticornis*	Woody debris	1/1996
E 268, tree R1; E 275, tree R1; E 276, tree R2; E 278, tree R2; E 280, tree R2; E 281, tree R2; E 283, tree R3; E 286, tree R4; E 287, tree R4; E 296, tree R6; E 297, tree R6; E 306, tree R9; E 307, tree R9; E 310, tree R9; E 312, tree R10; E 316, tree R14	Renmark, SA	*E. camaldulensis*	Woody debris	7/1998
E 1, E 1a, E 2, and E 3; tree Stl1	St Ives, Sydney, NSW	*A. costata*	Wood, detritus	10/1997 to 12/1997
E 444 (1 to 6); tree Stl1	St Ives, Sydney, NSW	*A. costata*	Woody debris	11/1998
E 7				
E 22, E 216, E 218, E 361, E 442	Coffs Harbour, NSW		Tree perch of koalas at zoo	12/1997 7/1998 9/1998 10/1998
E 71	Pilliga, NSW		Leaf litter	1/1998
E 147	Breza, NSW		Leaf litter	3/1998
E 238, E 383, E 388, E 389	Port Macquarie, NSW		Tree perch or ground of koalas at zoo	7/1998 10/1998
E 258	Port Macquarie, NSW	*E. grandis*	Woody debris	7/1998

[a] Supplied by D. Ellis and T. Pfeiffer, Mycology Unit, Women's & Children's Hospital, Adelaide, SA, Australia.

gattii DNA have indicated the presence of similar or identical *MF*α and *STE12*α genes in strains of the α mating type (30, 32).

The current study was undertaken to survey for the presence of the two mating types, mating types α and **a**, in clinical and environmental collections of *C. neoformans* var. *gattii* from different regions of Australia. As mating between isolates of *C. neoformans* var. *gattii* was difficult to induce, we used a molecular approach, using specific PCR primers to selectively amplify the *MF*α and *STE12*α sequences from α-mating-type strains.

MATERIALS AND METHODS

Collection of *C. neoformans* var. *gattii* isolates. One hundred thirty-one environmental isolates were obtained from various trees in three Australian states: South Australia (SA), New South Wales (NSW), and Queensland (QLD). The isolates were collected either from a single tree or from a number of trees (in close proximity). In addition, a few of the environmental isolates came from dead branches used as perches within a koala enclosure in a wildlife park. The details about these isolates are summarized in Table 1.

Thirty-nine isolates were obtained from animals with cryptococcosis within NSW and Western Australia (WA) (Table 2), and 30 clinical isolates from humans came from patients in the Northern Territory (NT), Victoria (VIC), SA, NSW, WA, and QLD, and were obtained from the culture collection of Westmead Hospital, Sydney, NSW (Table 3). For the isolates from animals, the month and year of infection were determined from the time when symptoms first became evident rather than from the time when the animal was presented to the veterinarian and *C. neoformans* var. *gattii* was cultured. The geographical locations of all environmental and clinical isolates are illustrated in Fig. 1.

All isolates were subcultured onto Sabouraud dextrose agar (Amyl medium) and were incubated for 48 h at 25°C for DNA extraction.

Mating type crosses. The reference strains B-3501 (mating type α) and B-3502 (mating type **a**) of *Filobasidiella neoformans* var. *neoformans* (10) and strains CBS 6956 (mating type α) and CBS 6955 (mating type **a**) of *F. neoformans* var. *bacillispora* (16) were used in all mating-type crosses. The media used for the crosses included V8 juice agar (14), sucrose biotin agar (11), eucalyptus seed agar (50 g of *E. camaldulensis* seed, 1 g of glucose, 1 g of KH_2PO_4, 1 g of creatinine, 15 g of Bacto Agar, 1,000 ml of distilled H_2O, 1 ml of penicillin G [20 U/ml], and 1 ml of gentamicin [80 mg/ml]), and moist autoclaved *E. camaldulensis* bark. A loopful of 2-day-old yeast cells of the strain being studied was mixed with a loopful of an α or **a** mating type tester strain in the center of the plate or bark (25), and the plate or bark was incubated at 25°C for 2 weeks. This was observed periodically for the development of the perfect state.

DNA isolation. The chromosomal DNA extraction procedure was based on the Novozyme 234, dodecyltrimethylammonium bromide, and hexadecyltrimethylammonium bromide method described by Wen et al. (29) with the following modifications: approximately 0.75 g of cells (wet weight) grown on Sabouraud dextrose agar were collected, the protoplasting solution was made with 10 mg of Novozyme 234 per ml of SCS buffer (20 mM sodium citrate, 1 M sorbitol), and all centrifugation steps were performed at 12,879 × *g*. The DNA pellet was resuspended in 100 μl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]) containing 10 μg of RNase A (Progen) per ml. DNA was diluted 1:10 for PCR amplification.

Primer design. The MFα primers (primers MFαU and MFαL) were designed from within the open reading frame of the *MF*α pheromone gene (20) and were expected to amplify a 109-bp fragment from α-mating-type strains. The STE12α primers (primers STE12αU and STE12αL) were designed from within the homeodomain of the *STE12*α gene (30) and were expected to amplify a 150-bp fragment from MATα strains. Primers 660U and 660L were designed from an anonymous DNA fragment amplified by randomly amplified polymorphic DNA (RAPD) analysis-PCR from a *C. neoformans* var. *gattii* strain. These primers were used in coamplifications with the MFα primers and were designed to amplify a 216-bp fragment from all *C. neoformans* var. *gattii* strains. Oligo 5.0 software was used to optimize the design of all primers. Primer sequences are listed in Table 4.

PCR amplification and sequencing. PCR amplifications were performed in 50-μl volumes containing 1× PCR buffer (0.1 M Tris-HCl [pH 8.3], 0.5 M KCl, 15 mM $MgCl_2$, 0.1% gelatin), 5% glycerol, 250 μM deoxynucleoside triphosphates, 2.5 U of *Taq* polymerase, 1 μl of diluted template DNA, and either 30 pmol of primers MFαU and MFαL plus 25 pmol of primers 660U and 660L or 25 pmol of primers STE12αU and STE12αL. The reaction mixtures were overlaid with sterile mineral oil (Sigma). Amplification conditions for PCR were 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55 or 50°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. All amplifications were carried

TABLE 2. Clinical isolates of *C. neoformans* var. *gattii* from animals

Isolate	Location	Animal source	Date (mo/yr) isolated
571 123	Windsor, Sydney	Alpaca	9–10/1995
571 118	Engadine, Sydney	Cat	1–02/1995
571 073	Coogee, Sydney	Cat	9–10/1992
571 015	Cobbity, Sydney	Cat	1–2/1990
571 058	Chiswick, Sydney	Cat	9–10/1991
571 111	Beacon Hill, Sydney	Cat	9–10/1994
571 115	Bondi Junction, Sydney	Cat	2/1995
571 108	Bayview, Sydney	Cat	7–8/1994
571 067	The Oaks, Sydney	Cat	3–4/1992
571 043	Hornsby Heights, Sydney	Cat	1–2/1991
571 112	Woollahra, Sydney	Cat	8/1994
571 093	Carlingford, Sydney	Dog	7–8/1982
571 116	Camden, Sydney	Dog	1–2/1995
571 094	West Wyalong, NSW	Dog	9–10/1995
571 098	Port Macquarie, NSW	Koala	3/1994
571 100	Taronga Zoo, Sydney	Koala	4/1994
571 146	Wentworthville, Sydney	Cat	1–2/1996
1408[a]	Perth, WA	Sheep	
1410[a]	Perth, WA	Sheep	
571 147	Rosemeadow, Sydney	Cat	5–6/1996
494[a]	Perth, WA	Horse	
WA 861	Perth, WA	Dog	4/1996
1409[a]	Perth, WA	Sheep	
571 170	Tamworth, NSW	Cat	5/1997
571 169	Fairfield, Sydney	Dog	4/1997
571 178	Rose Bay, Sydney	Cat	8/1997
571 171	Coffs Harbour, NSW	Koala	2/1997
571 172	Dubbo, NSW	Koala	3/1997
447/98	Newcastle, NSW	Cat	1998
Pilliga 1 to 10[b]	Pilliga, NSW	Koala	5/1998

[a] Supplied by D. Ellis and T. Pfeiffer, Mycology Unit, Women's & Children's Hospital, Adelaide, SA, Australia.
[b] Ten isolates.

out in a Perkin-Elmer Cetus model 480 Thermal Cycler. A total of 10 μl of each amplification product was electrophoresed at 10 V/cm and 40 mA in 2% agarose gels containing 0.50 ng of ethidium bromide per ml. The gels were visualized by UV transillumination and were photographed.

The PCR products of selected *C. neoformans* var. *gattii* and *C. neoformans* var. *neoformans* isolates were purified by polyethylene glycol 8000 precipitation (23) and were sequenced in both directions with a Perkin-Elmer model 377 automated sequencer with dye terminators and the MFα or STE12α primers. The sequences were edited and merged by using the TED (9) and SEQASM programs and were aligned by using CLUSTAL W (27). These programs were accessed through the Australian National Genomic Information Service at The University of Sydney.

Nucleotide sequence accession numbers. The GenBank accession numbers for the MFα sequences are AF 155335, AF 155336, AF 155337, AF 155338, AF 155339, AF 155340, and AF 155341. The GenBank accession numbers for the STE12α sequences are AF 155342, AF 155343, AF 155344, AF 155345, AF 155346, AF 155347, AF 155348, and AF 155349.

RESULTS

Mating-type crosses. The α and **a** mating type test strains of *F. neoformans* var. *neoformans* were observed to mate only on the V8 juice agar, producing a dense white mycelial phase at the edge of the yeast colony. Basidium-producing chains of basidiospores and hyphae with clamp connections were observed under the microscope. These strains did not mate on any of the other media tested.

The α and **a** mating type test strains of *F. neoformans* var. *bacillispora* did not mate on any of the media. Likewise, none of the clinical or environmental isolates included in this study reacted with the α or **a** mating type test strains of either variety or with other isolates from the same collection.

Coamplification with primers MFα and 660. The MFα primers successfully amplified a 109-bp fragment from all culture collection strains of both varieties known to be of the α mating type but did not produce a fragment from any of the strains of the **a** mating type. Coamplification with the MFα and 660 primers was performed with DNAs from all of the environmental and clinical isolates listed in Tables 1, 2, and 3. The CBS strains characterized as being of the α and **a** mating types were included in each PCR run to act as positive controls. Representative profiles are shown in Fig. 2 and 3a, and a complete summary of the ratio of α mating types:**a** mating types is given in Table 5.

Of the 69 clinical isolates coamplified by the MFα and 660 primers, one (571 093; Table 2) did not produce the MFα fragment. All clinical isolates produced the 660 fragment, although a slight variation in the size of this fragment was seen in some strains (Fig. 2). In contrast, 40 of the 131 environmental isolates did not produce the MFα band. These isolates were obtained from two of the nine locations sampled: Balranald (NSW) and Renmark (SA). Isolates Bal 3 (Table 1) and H26 (Table 3) did not produce the 660 band but did produce the MFα band.

The MFα fragment was sequenced from five *C. neoformans* var. *gattii* isolates and two *C. neoformans* var. *neoformans* isolates and had greater than 90% identity to the corresponding segment from the published *C. neoformans* var. *neoformans* *MF*α pheromone gene (Fig. 4a) (20).

Amplification with STE12α primers. The STE12α primers were designed to confirm the results already obtained from the MFα-660 coamplifications, as failure to produce the MFα band could also be due to a polymorphism(s) in the primer binding sites. Representative amplification profiles are shown in Fig. 3b. Previously characterized CBS strains of the α and **a** mating types were again included as positive controls. All iso-

TABLE 3. Clinical isolates of *C. neoformans* var. *gattii* from humans

Isolate[a]	Location	Clinical source	Date (mo/yr) isolated
H1	Adelaide, SA	CSF[b]	9/1990
H2	Adelaide, SA	CSF	6/1990
H3	Alice Springs, NT	CSF	
H4	Alice Springs, NT	CSF	9/1994
H5	WA		
H6	WA		
H7	WA		
H8	Alice Springs, NT	CSF	
H9	WA		
H10	WA		
H11	Darwin, NT		
H12	Townsville, QLD	CSF	8/1996
H13	Melbourne, VIC	Skin	12/1995
H14	Townsville, QLD	CSF	5/1997
H15	Townsville, QLD	CSF	7/1997
H16	Arnhem Land, NT	Lung	4/1996
H17	Alice Springs, NT	CSF	
H18	Newcastle, QLD	Skin	12/1994
H19	WA		
H20	Brisbane, QLD	CSF	5/1994
H21	Brisbane, QLD	Lung	12/1995
H22	Arnhem Land, NT	Lung	2/1994
H23	Brisbane, QLD	Lung	4/1994
H24	Brisbane, QLD	Lung	6/1995
H25	Sydney, NSW	CSF	5/1994
H26	Darwin, NT	CSF	1/1994
H27	Toowomba, QLD	CSF	12/1994
H28	Sydney, NSW	Lung	12/1994
H29	Melbourne, VIC	Blood	1991
H30	Melbourne, VIC	Lung	1988

[a] All isolates supplied by W. Meyer and H. Daniel, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, NSW, Australia.
[b] CSF, cerebrospinal fluid.

lates that did not produce the MFα band also failed to produce the STE12α band. All remaining isolates produced both bands. Direct sequencing of the STE12α PCR fragments from five *C. neoformans* var. *gattii* isolates and three *C. neoformans* var. *neoformans* isolates found that they had a high degree of homology to the corresponding segment of the published *STE12*α gene, with polymorphisms shared between isolates belonging to each variety (Fig. 4b) (30).

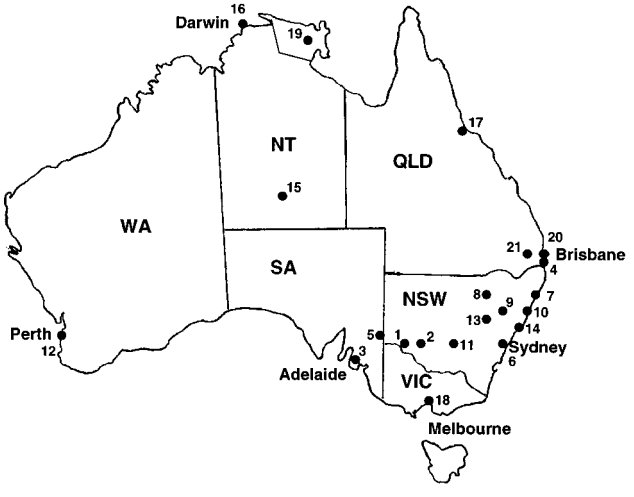

FIG. 1. Distribution of environmental and clinical isolates used in the study: 1, Balranald; 2, Hay; 3, Adelaide; 4, Gold Coast; 5, Renmark; 6, Sydney; 7, Coffs Harbour; 8, Pilliga; 9, Breza/Tamworth; 10, Port Macquarie; 11, West Wyalong; 12, Perth; 13, Dubbo; 14, Newcastle; 15, Alice Springs; 16, Darwin; 17, Townsville; 18, Melbourne; 19, Arnhem Land; 20, Brisbane; 21, Toowomba.

TABLE 4. Primers used in PCR amplifications

Primer	Sequence (5′ to 3′)	Annealing temp (°C)
MFαU	TTCACTGCCATCTTCACCACC	55
MFαL	TCTAGGCGATGACACAAAGGG	55
660U	TATTGGACTAAAACGGTATGCGG	55
660L	CGACGACGAGGTATTCTTTTTCC	55
STE12αU	CAATCTCAAAGCGGGGACAG	50
STE12αL	CTTTGTTTCGGTCCTAATACAGCC	50

DISCUSSION

For more than a decade, *C. neoformans* var. *gattii* has been known to have a specific ecological association with a number of eucalyptus species. Epidemiological studies have supported the association between human clinical disease and the natural reservoir of the fungus (24). The current study was undertaken to survey for the presence of the α and **a** mating types in *C. neoformans* var. *gattii* strains isolated from the environment and from humans and animals with cryptococcosis. The eventual aim of this work will be to determine whether the fungus completes its life cycle in association with the eucalyptus host, undergoing sexual recombination and producing potentially infectious basidiospores.

Mating analyses failed to determine the mating type of any of the isolates used in this study, despite the use of a variety of different isolates and a range of conditions. Kwon-Chung and colleagues (17) experienced similar difficulties with inducing mating in four strains of *C. neoformans* var. *gattii* isolated from *E. camaldulensis* trees. We therefore used a molecular approach to determine mating type.

To date, only the α-mating-type locus has been isolated and sequenced, and we were able to assign the **a** mating type to an isolate only by failure to amplify the α-mating-type-specific sequences. A positive control was therefore included in the MFα amplifications to ensure that the absence of the MFα fragment was not due to inhibition of the PCR. Two isolates, isolates H26 and Bal 3, did not produce a band with the

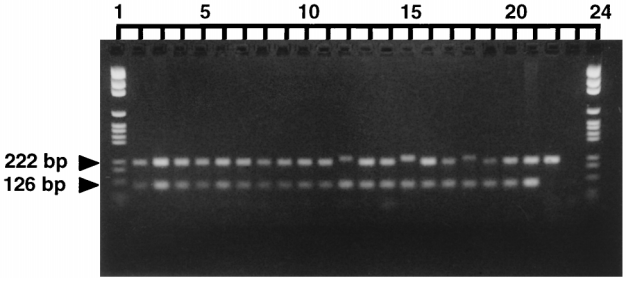

FIG. 2. Representative gel of DNA from clinical and environmental *C. neoformans* var. *gattii* isolates coamplified with the MFα and 660 primers. Lanes: 1 and 24, pGEM size marker (Promega); 2, GC5; 3, GC12; 4, GC17; 5, GC22; 6, GC27; 7, Ad5; 8, Ad12; 9, Ad17; 10, Ad22; 11, Ad26; 12, 1408; 13, 571 146; 14, 571 067; 15, 1409; 16, 571 178; 17, H28; 18, H23; 19, H13; 20, H8; 21, CBS 5757 (α); 22, CBS 6998 (**a**); 23, negative control for PCR amplification. The upper band of 216 bpl of 109 bp was amplified by the 660 primers; the lower band was amplified by the MFα primers. Primer dimer can be seen below many of the amplified fragments. Lanes 12, 15, and 18 show slight variation in size of 660 fragment.

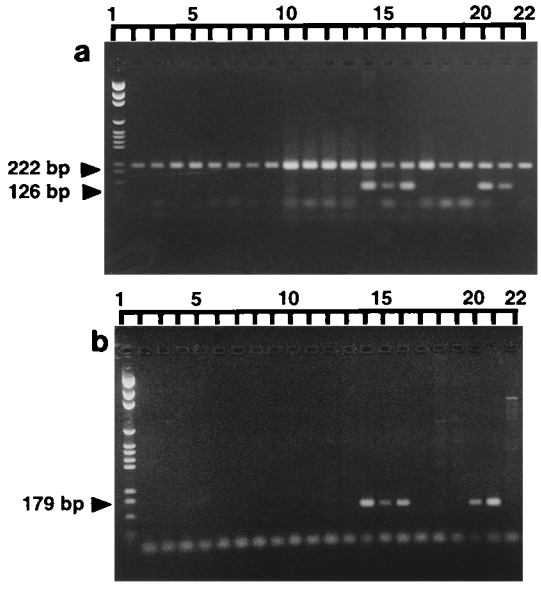

FIG. 3. Representative gel of DNA from Balranald isolates coamplified by the MFα and 660 primers (a) and the STE12α primers (b). Lanes 1: pGEM size marker; 2, Bal 23; 3, Bal 24; 4, Bal 25; 5, Bal 26; 6, Bal 27; 7, Bal 28; 8, Bal 29; 9, Bal 30; 10, 401 Bal 2; 11, 402 Bal 6; 12, 402/1; 13, 403 Bal 8; 14, 405 Bal 2c; 15, 406 Bal 2d; 16, 407 Bal 2f; 17, 408 B1; 18, 409 B2; 19, 410 B5; 20, 404 H22b; 21, CBS 5757 (α); 22, CBS 6998 (**a**). Primer dimers can be seen in many of the lanes.

positive control 660 primers, but amplification was successful with the MFα primers. RAPD analysis-PCR has since shown Bal 3 to be *C. neoformans* var. *neoformans* (data not shown), but isolate H26 is definitely *C. neoformans* var. *gattii* as it is serotype B. It is therefore likely that this isolate has a polymorphism at one of the 660 primer binding sites. In addition, slight differences in the sizes of the product amplified by the 660 primers were seen between some of the clinical isolates (Fig. 2). These isolates have been found to be of the VGII type, a minor variant of *C. neoformans* var. *gattii* previously reported by Sorrell et al. (24).

A total of 100% of the human and 97.4% of the animal clinical isolates produced both the MFα band and the STE12α band and can therefore be assumed to be of the α mating type. This imbalance is similar to that found in studies of clinical isolates of *C. neoformans* var. *neoformans* (12, 19, 26) and may indicate that, like *C. neoformans* var. *neoformans*, *C. neoformans* var. *gattii* α isolates are more virulent than **a** isolates and are therefore more likely to infect humans and animals (15). The only MAT**a** clinical isolate, 571 093, came from a dog with respiratory failure from which *C. neoformans* var. *gattii* was cultured from the lower respiratory tract. No unusual or atypical symptoms were noted in this cryptococcal infection.

Of the 131 environmental isolates of *C. neoformans* that were collected to determine their mating types, 130 were *C. neoformans* var. *gattii*, and 91 (70%) of these were of the α mating type. This is substantially lower than the result of a similar study with *C. neoformans* var. *neoformans*, in which 97.5% of the isolates were of the α mating type (12); however, the current study may have been biased by the inclusion of many isolates from single trees. Of the 31 isolates taken from separate trees, 80.6% were of the α mating type. This is still less than the proportion for *C. neoformans* var. *neoformans*, although the α mating type remains predominant. In *C. neoformans* var. *neoformans*, the bias in mating type has been postulated to be due to haploid fruiting. In this process, haploid α cells form extensive hyphae in the absence of the opposite mating type, producing abundant blastospores and basidia bearing viable basidiospores that are all of the α mating type. This has also been observed in *C. neoformans* var. *gattii* strains, but the hyphae are less extensive, and while blastospores are produced, no basidiospores have been detected. Haploid fruiting apparently cannot occur in **a**-mating-type strains of either variety (31).

MAT**a** isolates were found in collections from two areas, Renmark and Balranald, which lie less than 300 km apart and which share very similar geographies and floras. All the Balranald isolates came from two separate samplings from an individual tree. This tree was unique in that the *C. neoformans* var. *gattii* isolates obtained from it were overwhelmingly of the **a** mating type, especially those isolated in 1996 (27 of 29). These isolates came from a large amount of debris in a semihollow area located at the base of the tree. When isolates taken in 1989 and 1990 were examined, 7 of 10 isolates were of the **a** mating type, indicating that the predominance of **a**-mating-type isolates in 1996 may have been caused by sampling artifact. Alternatively, the **a** mating type may be becoming more predominant over time via clonal propagation. We are currently using RAPD analysis-PCR to assess the genetic diversity in this collection.

The results presented in this report indicate that, in contrast to *C. neoformans* var. *neoformans*, both mating types can be found in some populations of *C. neoformans* var. *gattii*. In particular, in the population from Renmark, the ratio of the two mating types (10 α:6 **a**) approximated the 50:50 ratio expected to result from sexual outcrossing. The ability of pathogens to recombine sexually is important for their ability to survive the host immune response and to adapt to novel environmental challenges, including antimicrobial therapy. The clonal population structure reported for *C. neoformans* var. *neoformans* (7) is unusual compared to those reported for other medically important fungi, in which a history of sexual exchange has been seen (1, 2). Our results suggest that *C. neoformans* var. *gattii* may also reproduce sexually, but further studies analyzing the association of molecular markers are

TABLE 5. Summary of mating types among 130 environmental and 69 clinical isolates of *C. neoformans* var. *gattii*

Source	α mating type:**a** mating type	% α mating type
Single tree		
Adelaide	26:0	100
Balranald, 1989–1990	3:7	30
Balranald, 1996	2:27	7
Gold Coast	25:0	100
Separate trees		
Breza	1:0	100
E 7	1:0	100
Hay	1:0	100
Pilliga	1:0	100
Port Macquarie	1:0	100
Renmark	10:6	62.5
St Ives	10:0	100
Animal cage		
Coffs Harbour	5:0	100
Port Macquarie	4:0	100
Clinical isolates		
Animal	38:1	97.4
Human	30:0	100

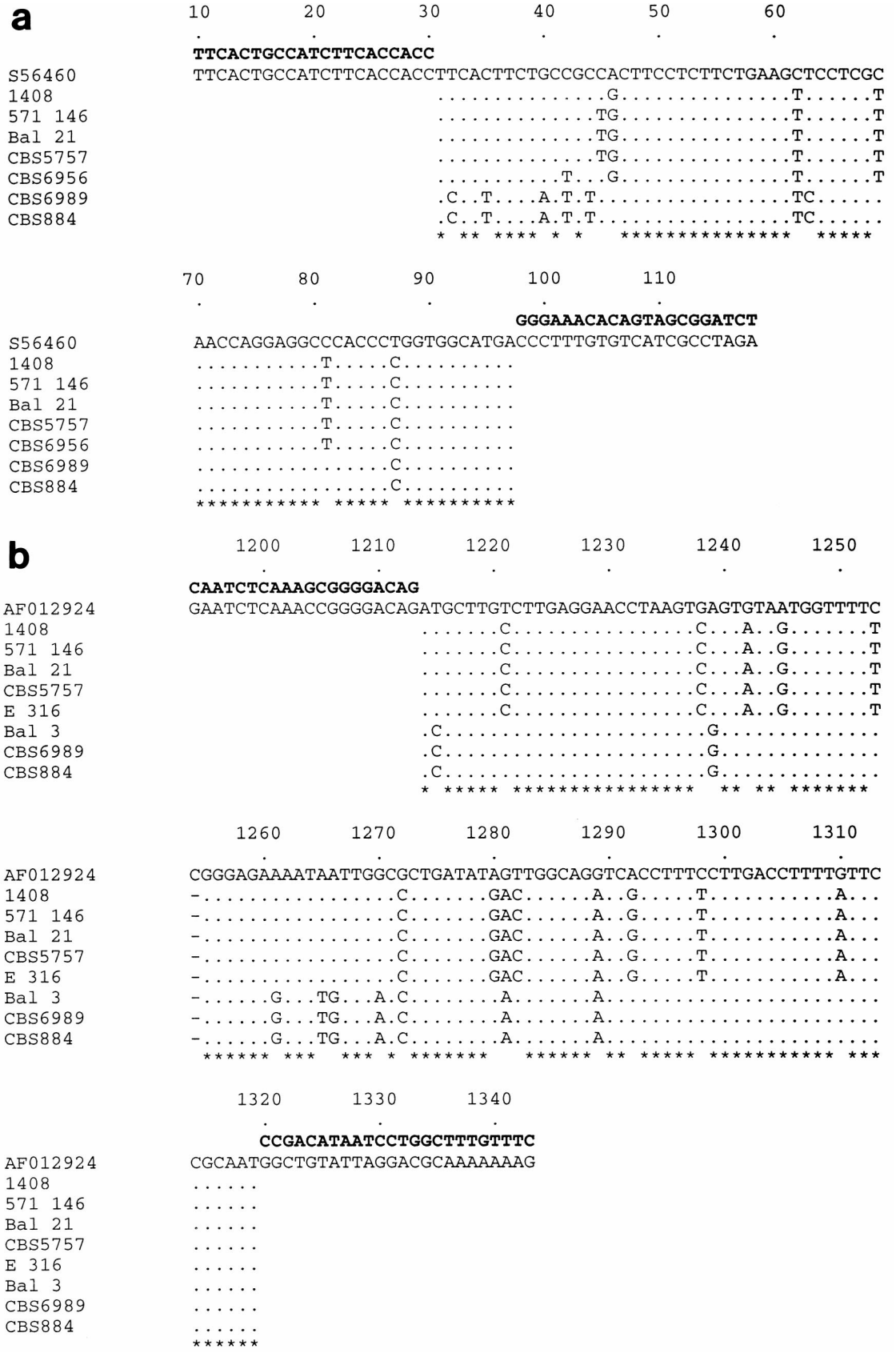

FIG. 4. Sequence alignments of the 109-bp MFα fragment with the *MFα* gene (GenBank accession no. S56460), which covers nucleotide positions 10 to 118 (a), and the 149-bp STE12α fragment with the corresponding segment of the STE12α GenBank sequence (accession no. AF012924), which covers nucleotide positions 1194 to 1343 (b). Both GenBank sequences were from *C. neoformans* var. *neoformans* isolates. The primer sequences are shown in bold, and asterisks indicate positions which are identical in all isolates.

necessary to test this hypothesis (28). The mating type survey indicates that Renmark will be a suitable region as a target for future studies of genetic recombination in *C. neoformans* var. *gattii*.

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