Clinical and Environmental Isolates of Cryptococcus gattii from Australia That Retain Sexual Fecundity†

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Cryptococcus gattii is a primary pathogenic yeast that causes disease in both animals and humans. It is closely related to Cryptococcus neoformans and diverged from a common ancestor ~40 million years ago. While C. gattii has a characterized sexual cycle dependent upon a dimorphic region of the genome known as the MAT locus, mating has rarely been observed in this species. In this study, we identify for the first time clinical (both human and veterinary) and environmental isolates from Australia that retain sexual fecundity. A collection of 120 isolates from a variety of geographic locations was analyzed for molecular type, mating type, and the ability to develop mating structures when cocultured with fertile tester strains. Nine isolates produced dikaryotic filaments with paired nuclei, fused clamp connections, and basidiospores. DNA sequence analysis of three genes (URA5, the MATα-specific SXI1α gene, and the MATα-specific SX2α gene) revealed little or no variability in URA5 and SX2α, respectively. However, across the 108 MATα strains sequenced, the SX11α gene was found to exist as 11 different alleles. Phylogenetic analysis found most variation to occur in the more fertile genotypes. Although some lineages of Australian C. gattii have retained the ability to mate, the majority of isolates were sterile, suggesting that asexuality is the dominant mode of propagation in these populations.

While many organisms undergo sexual recombination, others have limited sexual ability or appear to lack sex entirely. Sex can increase the rate of adaptive evolution by creating offspring with favorable gene combinations that are more robust than those of their parents in changing environments, and sex can also limit the accumulation of deleterious mutations by shuffling unfavorable gene combinations (2). However, sex can also disturb beneficial gene combinations and can be disadvantageous when a proportion of the population does not contribute to the fecundity of the opposite mating type or sex. For example, investing resources in the development of males not utile to the fecundity of the opposite mating type or sex, for instance, mating structures when cocultured with fertile tester strains. Nine isolates produced dikaryotic filaments with paired nuclei, fused clamp connections, and basidiospores. DNA sequence analysis of three genes (URA5, the MATα-specific SXI1α gene, and the MATα-specific SX2α gene) revealed little or no variability in URA5 and SX2α, respectively. However, across the 108 MATα strains sequenced, the SX11α gene was found to exist as 11 different alleles. Phylogenetic analysis found most variation to occur in the more fertile genotypes. Although some lineages of Australian C. gattii have retained the ability to mate, the majority of isolates were sterile, suggesting that asexuality is the dominant mode of propagation in these populations.

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morphisms detected by PCR fingerprinting, randomly amplified polymorphic DNA, and restriction fragment length polymorphism (RFLP) analyses (29, 36). Serotype A isolates produce VNI or VNIII fingerprints, AD hybrids produce a VNIII pattern, and serotype D isolates produce a VNI pattern. Serotypes B and C define the groups VGI to VGV (30).

Mating was first reported in *C. gattii* over 25 years ago (21). This species is endemic in the Australian environment and can be isolated in high numbers from certain native Australian eucalyptus trees (4, 7, 19). Although the mating type of either species was generally mixed populations of α and α mating types have been observed in one Australian sampling site (14). Population genetics analysis revealed no apparent meiotic recombination among isolates, however, and it was not possible to induce mating in the laboratory with any of the known tester strains. It was suggested that the eucalyptus tree may simply be a favorable substrate for clonal propagation, and a different primary ecological niche might support the complete fungal life cycle (13). Recently, mating was recapitulated in several *C. gattii* isolates; however, mating had yet to be observed in any environmental isolates of *C. gattii* from Australia (9).

In this study, 120 Australian *C. gattii* isolates were assayed for mating ability using serotype C tester strains recently developed to induce sex in this species (9). We report the first mating of confirmed Australian *C. gattii* isolates and provide biological evidence supporting the model that mating ability is linked to the VGII molecular type, an observation of particular import given that the fertile Australian isolates and the Vancouver outbreak isolates share this characteristic.

**Materials and Methods**

**Strains and media.** All strains used in this study are listed in the supplemental material (see Table S1 in the supplemental material). Reference strains used were the congeneric serotype D strains JE20 (MATα) and JEC21 (MATα), serotype B strains JER66 (MATα) and WM276 (MATα), serotype C strains B4546 (MATα) and NIH312 (MATα), and the serotype C *C. albicans* mutants derivatives JF109 (MATα) and JF101 (MATα). All strains were maintained on yeast extract-peptone-dextrose medium. Isolates were originally serotyped at the source laboratories, and serotypes were confirmed using the Crypto Check kit (Iatron Laboratories Inc., Tokyo, Japan).

**Mating assays.** The ability to mate was tested using the serotype D tester strains JEC20 (MATa) and JEC21 (MATα), serotype C strains B4546 (MATα) and NIH312 (MATα), and their congenic *C. albicans* mutants derivatives JF109 (MATα) and JF101 (MATα) (9). Strains were pregrown on yeast extract-peptone-dextrose medium for 2 days and then cultured on V8 medium (5% [vol/vol] V8 juice, 3 mM KH2PO4, 4% [wt/vol] agar, pH 7?), both alone and mixed with a tester strain, at room temperature for 21 days in the dark and assessed for filament and basidiospore formation via light microscopy. Similarly, monokaryotic fruiting was assessed by culturing isolates alone or adjacent to co-cultured MATa/MATα pheromone donors on V8 medium at pH 7 at room temperature in the dark for 21 days.

**Determination of mating type.** *C. gattii* genomic DNA for PCR analysis was prepared as described previously by Pitkin et al. (33). Mating type was determined by PCR using primers specific for the STE12a, STE20a, MFα1, and MFα2 genes (Table 1). The STE12a and MFα1 primers, respectively, amplified 148-bp and 196-bp fragments specific to the α mating type. The STE20a and MFα2 primers amplified 484-bp and 213-bp fragments, respectively, specific to the α mating type. Ten microclones of each amplification product was electrophoresed on 2% agarose Tris-acetate-EDTA (TAE) gels and visualized by UV transillumination. RFLP analysis employed a method modified from that described previously by Meyer et al. (29). The URA5 gene was amplified using primers J0H13344 and J0H13345 (Table 1) and digested with Sau96I and HhaI for 3 h, and restriction fragments were resolved on 3% agarose TAE gels. Molecular type standard strains were included as controls and used to group sample isolates (Table 2).

**DNA sequencing.** The URA5 gene was sequenced from all isolates. The SXI1 gene was sequenced from MATa strains, and the SXI2 gene was sequenced from the MATa strains. PCR products were purified and sequenced using an ABI 3700 sequencer with Big Dye chemistry (Applied Biosystems) and assembled using Sequencher 3.1.2 (Gene Codes). Sequencing primers are listed in Table 1. Nucleotide sequences were translated using the program Translate (ANGIS, Biomanager), and all sequences were aligned using ClustalW (41). The phylogenetic analysis program PAUP+ (version 4.0b4a) (39) was used to produce a parsimony phylogram from sequence alignments of the SXI1 alleles. The same genes were also sequenced from the control strains WM276, E566, NIH312, and B4546.

**Nucleotide sequence accession numbers.** Representative strains of each allele identified for the URA5, SXI1α, and SXI2α genes have been deposited in GenBank under accession numbers AY973637 to AY973640 for URA5 alleles A to D, AY973641 to AY973651 for SXI1α alleles A to D, B to D, C to D, and AY973652 to AY973654 for SXI2α alleles A to C, respectively.

**Results**

**Molecular characterization of *C. gattii* isolates from Australia.** A total of 120 strains from distant geographic locations around Australia were collected, some as part of a concurrent population genetics-based effort to identify evidence of sexual recombination in *C. gattii* (Fig. 1; see Table S1 in the supplemental material). This included 55 environmental, 17 human clinical, and 48 veterinary clinical isolates (see Table S1 in the supplemental material). These isolates were analyzed for serotype, molecular type, mating type, ploidy, and mating ability.

Analysis using the Crypto Check slide agglutination test, which separates isolates into serotypes A, B, C, D, and AD by virtue of different capsular antigens, revealed that all isolates in the collection were *C. gattii* serotype B. Isolates were further differentiated by molecular typing. PCR fingerprinting using the microsatellite DNA primer (GACA)4 and RFLP analysis of the URA5 gene yielded concordant results and revealed three of the four previously established *C. gattii* genotypes groups, namely, VGI, VGII, and VGIII. From the 120 isolates assayed, 97 were VGI, comprising 93% of the environmental isolates, 29% of the human clinical isolates, and 88% of the veterinary isolates. Of the remaining 23 isolates, 22 were the VGII molecular type, and this comprised 7% of the remaining environmental isolates, 71% of the human clinical isolates, and 10% of the veterinary isolates. A single veterinary isolate was molecular type VGIII.

The mating type of each isolate was determined with a PCR-based protocol using both MATa- and MATα-specific primers (Table 1). MATa strains outnumbered MATα strains by a ratio of 113:7, which correlates well with previous studies of mating type distribution in Australia and elsewhere (14, 28).
No a/α diploid or aneuploid isolates were observed, based on the absence of strains with both MATα and MATα PCR products.

The seven MATα isolates were all molecular type VGI. Of these, four originated from the environment, two were human clinical isolates, and one was a veterinary isolate. Of the MATα/H9251 isolates, the environmental isolates (n/H11005/51) were 92.2% VGI

### Table 1. Primers used in this study

| Primer | Primer designation | Sequence | Reference or source |
|--------|--------------------|----------|---------------------|
| STE12α upper | JOHE8644 | CAATCTCAAGGCGGGAGACAG | 9 |
| lower | JOHE8645 | CTTTGTTTCGTCCTAATACGCC | |
| STE20α upper | JOHE9421 | ATCCGCCTCGAATGCCA | 9 |
| lower | JOHE9422 | TGGCGACGACTGTGAGAT | |
| MFα upper | JOHE9779 | CCTTACTCAATAACCGCTAC | 9 |
| lower | JOHE9780 | AATTTCTAAGCCTTGGGAA | |
| MFα (GACA)a upper | JOHE9787 | ACACCGCTGTATCCAAATGGAC | 9 |
| lower | JOHE9788 | CAGGCTTACGAGATTTACCTT | |
| URA5 (RFLP) SJO1 | JOHE10455 upper | CGATACTAAGGCGTCCGAT | This study |
| lower | JOHE10456 lower | CCGAAGTCACTGATACACC | This study |
| lower | JOHE10551 lower | CACGCTTCAAGTCTGTCAG | This study |
| SXI1α upper | JOHE10451 upper | TACATCCGAGTCATATTCG | This study |
| lower | JOHE10452 lower | TCTGGGAAACGCGCTACTGGA | This study |
| lower | JOHE10553 lower | CGCCGCTCGTCTTGGTCTGTC | This study |
| lower | JOHE10554 lower | CCCATGTCGTCCGCTCCTC | This study |
| lower | JOHE10555 lower | AGAAGGCGACGACACTTCC | This study |
| SXI2α upper | JOHE10453 upper | TGATCGACGAGCCAAATCCC | This study |
| lower | JOHE10454 lower | GGCCTTCGTGAAACACTTCTA | This study |
| lower | JOHE10556 lower | TGCACCAGGCTCGCTACTTCA | This study |
| lower | JOHE10557 lower | TGGAGATAACATACCGGGGCA | This study |
| lower | JOHE10558 upper | CGCACCACAGCGTCTTCTTA | This study |
| lower | JOHE10559 lower | TGAAGGAAGCAATGCGTGGCG | This study |
| lower | JOHE10560 upper | AATGTTCCGGCGGCGATTTCTC | This study |
| lower | JOHE10561 lower | GAGAACATCGGCCAAACTT | This study |
| lower | JOHE10562 upper | TGGAGTCTTCGCTCCTCC | This study |
| lower | JOHE10563 lower | CGTAGGGAGGCAAAACTCTCA | This study |

### Table 2. Control and test strains used in this study

| Strain | Serotype | Mating type | Molecular type | Source |
|--------|----------|-------------|----------------|--------|
| JEC20  | D        | a           | VNIV           | K. J. Kwon-Chung |
| JEC21  | D        | α           | VNIV           | K. J. Kwon-Chung |
| CDCB4546 | C   | a           | VGIII          | W. Schell |
| NIH312 | C        | α           | VGIII          | K. J. Kwon-Chung |
| JF109 (CDCB4546 crg1::NEO) | C | α | VGIII | J. Heitman |
| JF101 (NIH312 crg1::NAT) | C | α | VGIII | J. Heitman |
| E566   | B        | a           | VGI            | M. Krokenberger |
| WM276  | B        | α           | VGI            | W. Meyer |
| JF4 (E566 crg1::NAT) | B | a | VGI | J. Heitman |
| JF7 (WM276 crg1::NEO) | B | α | VGI | J. Heitman |
| Byron WM179 | B | α | VGI | W. Meyer |
| Cotter WM178 | B | α | VGI | W. Meyer |
| 689 WM161 | B | α | VGI | W. Meyer |
| King Cheetah WM779 | B | α | VGI | W. Meyer |

FIG. 1. Regional map of Australia showing source locations of Australian isolates. The white arrow indicates the Sydney region primary location for veterinary isolates; dark and light gray arrows indicate the Renmark/Balranald and Western Australian regions, respectively, sources of many environmental isolates; the black arrow indicates the Northern Territory and Arnhemland, where the clinical isolates and some environmental isolates were obtained. Scale bar is 1,000 km.

TABLE 2. Control and test strains used in this study
and 7.8% VGII, the human clinical isolates (n = 15) were 20% VGI and 80% VGII, and the veterinary isolates (n = 47) were 87.2% VGI, 10.6% VGII, and 2.1% VGIII.

Fertility of Australian C. gattii isolates. Mating tests used the congeneric serotype D strains JEC20 (MATα) and JEC21 (MATα) (21), robustly mating serotype C strains B4546 (MATα) and NIH312 (MATα), and their crg1 mutant derivatives JF109 (MATα crg1Δ) and JF101 (MATα crg1Δ) (9). CRG1 encodes an RGS (regulator of G protein signaling) protein that attenuates pheromone signaling, and as a consequence, the crg1 mutation enhances pheromone response and mating (31, 44). Matings ability for each test isolate was determined by coculturing sample isolates with tester strains on V8 medium in the dark at room temperature for 21 days. Evidence of typical mating structures, including filaments, basidia, and basidiospores, was examined by microscopy. Isolates were considered fertile if they developed any of these structures and were considered robustly fertile if basidiospores were formed.

Of the 120 isolates tested, 27 were observed to produce mating structures when mixed with inter- and/or intravarietal tester strains (Table 3); 18 of these isolates developed filaments and basidia, and 9 isolates developed filaments, basidia, and long chains of basidiospores extending from the basidia (Fig. 2). The remaining 93 isolates showed no mating response. Matings ability varied among strains of different origin. This phenotype was seen in a total of 23% (n = 27) of isolates, which included 11% (n = 6) of the environmental isolates, 71% (n = 12) of the human clinical isolates, and 19% (n = 9) of the veterinary isolates. A bias towards the VGII molecular type was observed among the fertile isolates, and this was linked to the higher proportion of this type observed among the human clinical isolates. Complete concordance was seen between their response to the MATα and MATa tester strains and the determination of mating type by PCR analysis. The ratio of MATα to MATa in the fertile collection, 26:1 (n = 27), again reflects the bias toward α strains in C. gattii (Table 4) (14, 22, 25, 28).

All isolates that produced basidiospores and that were considered to be robustly fertile were MATα and molecular type VGII (eight human clinical isolates and one veterinary isolate). A similar pattern has been seen in the ongoing Vancouver Island outbreak, in which >99% of isolates are molecular type VGII, are fertile, and produce basidiospores, and all typeable isolates are MATα (9, 18) (J. A. Fraser and J. Heitman, unpublished data). This correlation suggests that a link may exist between molecular type, the increased rate of infection as seen in the Vancouver Island population (10), and the relatively high levels of infection in northern Australia by C. gattii, which are also frequently due to VGII strains (4, 5) (L. T. Campbell et al. this issue).

C. gattii produced dikaryotic filaments with fused clamp connections. In addition to mating, C. gattii could potentially

### Table 3. List of 27 fertile Australian isolates

| Isolate designation | Isolate name (original) | Source (host) | Serotype | Mating type | Mating ability | DNA sequencing | Molecular type | Source |
|---------------------|-------------------------|--------------|----------|-------------|----------------|----------------|----------------|--------|
| JH3726              | NT-9                    | Clinical (human) | B α R | + + + + | D Biia NA VGII 1 |
| JH3732              | NT-11                   | Clinical (human) | B α R | + + + + | C Biia NA VGII 1 |
| JH3734              | NT-13                   | Clinical (human) | B α R | + + + + | C Biia NA VGII 1 |
| JH3739              | RDH-2                   | Clinical (human) | B α R | + + + + | D C NA VGII 1 |
| JH3743              | RDH-6                   | Clinical (human) | B α R | + + + + | C Biia NA VGII 1 |
| JH3744              | RDH-7                   | Clinical (human) | B α R | + + + + | D Biia NA VGII 1 |
| JH3745              | RDH-9                   | Clinical (human) | B α R | + + + + | D Biia NA VGII 1 |
| JH3631              | 57I08 or V4             | Clinical (vet-cat) | B α R | + + + + | C Biia NA VGII 5 |
| JH3729              | NT-8                    | Clinical (human) | B α R | + + + + | C Biia NA VGII 5 |
| JH3727              | NT-7                    | Clinical (human) | B α R | + + + + | C Biia NA VGII 5 |
| JH3733              | NT-12                   | Clinical (human) | B α I | + + + + | C Biia NA VGII 5 |
| JH3622              | WA861                   | Clinical (vet-koala) | B α I | + + + + | C Biia NA VGII 5 |
| JH3628              | V21                     | Clinical (vet-cat) | B α I | + + + + | C Biia NA VGII 5 |
| JH3629              | V9                      | Clinical (vet-cat) | B α I | + + + + | C Biia NA VGII 5 |
| JH3637              | 57I116 or V20           | Clinical (vet-dog) | B α I | + + + + | C Biia NA VGII 5 |
| JH3643              | VPB 57I159              | Clinical (vet-cat) | B α I | + + + + | C Biia NA VGII 5 |
| JH3715              | V5 or 57I063            | Clinical (vet-cat) | B α I | + + + + | C Biia NA VGII 5 |
| JH3715              | V11 or 57I083           | Clinical (vet-dog) | B α I | + + + + | C Biia NA VGII 5 |
| JH3720              | V26 or 57I147           | Clinical (vet-cat) | B α I | + + + + | C Biia NA VGII 5 |
| JH3725              | NT-4                    | Clinical (human) | B α W | + + + + | A NA B VGII 1 |
| JH3738              | Arn1                    | Environmental   | B α W | + + + + | C Biia NA VGII 1, 2 |
| JH3740              | Ram2                    | Environmental   | B α W | + + + + | C Biia NA VGII 1, 2 |
| JH3741              | Ram5                    | Environmental   | B α W | + + + + | C Biia NA VGII 1, 2 |
| JH3742              | Ram15                   | Environmental   | B α W | + + + + | C Biia NA VGII 1, 2 |
| JH3661              | Env71                   | Environmental   | B α W | + + + + | A Av NA VGII 3, 4 |
| JH3665              | Env1a                   | Environmental   | B α W | + + + + | A Av NA VGII 3, 4 |

\(^a\) Isolates are listed in descending order from robust to weak mating ability. Mating ability was considered robust (R), intermediate (I), or weak (W). Mating was performed with V8 agar for 21 days at 25°C.

\(^b\) Source numbers correspond to the following individuals: Bart Currie, Infectious Diseases Program, Menzies School of Health Research and Northern Territory Clinical School, Royal Darwin Hospital, Northern Territory, Australia (1); David Ellis, Mycology Unit, Women’s and Children’s Hospital, Adelaide, South Australia, Australia (2); Catriona Halliday, Department of Microbiology, University of Sydney, New South Wales, Australia (3); Mark Krookenberger, Faculty of Veterinary Science, University of Sydney, New South Wales, Australia (4); and Richard Malk, Faculty of Veterinary Science, University of Sydney, New South Wales, Australia (5).

\(^c\) Vet, veterinary isolate.
engage in two other forms of filamentous differentiation: monokaryotic fruiting in haploid strains and self-filamentous growth in a/α diploid strains (26). Each of these developmental processes produces basidiospores, and each is characterized by hallmark morphological features. Mating can be distinguished by the presence of dikaryotic filaments with paired nuclei and fused clamp connections as opposed to the monokaryotic filaments and unfused clamps seen in monokaryotic fruiting. Isolates capable of producing basidiospores were crossed with tester strains, cultured on glass slides, and fixed. The cell wall was stained with calcofluor white, DNA in the nuclei was stained with Sytox green, and the filaments were examined by DIC microscopy. All cocultures produced dikaryotic filaments with fused clamp connections and therefore represent bona fide mating (Fig. 3).

_C. gattii_ diploid a/α isolates developed in the laboratory are thermally dimorphic and at lower growth temperatures produce filaments and sporulate (9). To exclude the possibility that the mating observed here was due to the presence of such isolates, FACS analysis was performed on subsets of mating and nonmating isolates. All sample isolates had a haploid profile when compared to control strains of known ploidy (data not shown). This was further confirmed by the PCR determination of mating types, which found only the a or α mating locus allele to be present. Furthermore, to our knowledge, no diploid _C. gattii_ isolates have been identified from any natural source.

Monokaryotic fruiting was not observed in Australian _C. gattii_ isolates. In this study, no monokaryotic fruiting was observed for any Australian strain. These results indicate that the Australian isolates tested are incapable of monokaryotic fruiting under conditions that evoked a mating response in competent isolates. However, under these conditions, monokaryotic fruiting, including the development of basidiospores, was observed in the _C. neoformans_ serotype D tester strain JEC21, which acted as a positive control. As an extension of this assay, all mating-competent strains of the same mating type were crossed pairwise with each other on V8 medium and examined. No mating structures (enlarged cells or conjugation tubes) or fruiting structures were observed, indicating that mating was restricted to interactions between strains of opposite mating types.

![Image of mating morphology](image-url) **FIG. 2. Mating morphology of Australian _C. gattii_ isolates.** Crosses of Australian _C. gattii_ strains with the serotype C _crg1_ mutant and wild-type strains and serotype D tester strains are shown. Morphology was observed by light microscopy. (A) 3739 (human clinical) × JF109; (B) 3739 × CDCB4546; (C) 3739 × JEC20. All fertile isolates produced mating structures with the _crg1_ mutant tester strains. Panel A illustrates the robust mating observed in 33.3% of fertile isolates when cocultured with the corresponding serotype C _crg1_ mutant strain. These crosses produced long chains of oval-shaped basidiospores. Panel B demonstrates the weaker mating observed in crosses with the wild-type serotype C tester strains in which fewer filaments and basidia developed and some basidia failed to produce basidiospores. Panel C illustrates the sexual structures developed in intervarietal crosses. Scale bars are 100 μm (panel) and 10 μm (insert).

| Population (total no. of isolates) | No. of fertile isolates | No. with molecular type: | No. with mating type: |
|-----------------------------------|-------------------------|--------------------------|----------------------|
| Environmental (55)               | 6                       | 2                        | 4                    | 0                    | 6                     | 0                     |
| Human clinical (17)               | 12                      | 1                        | 11                   | 0                    | 11                    | 1                     |
| Veterinary (48)                   | 9                       | 2                        | 6                    | 1                    | 9                     | 0                     |
| Total (120)                       | 27                      | 5                        | 21                   | 1                    | 26                    | 1                     |
DNA sequence analysis of *URA5*, *SXI1a*, and *SXI2a* genes.

Sequence analysis was conducted to determine whether genetic variation occurred between fertile and infertile *C. gattii* isolates. Of the total 120 isolates in the study, 115 were included in the sequence analysis. *URA5* sequences divided the isolates into three major allelic groups that correspond to the three VG groups. Five VGII isolates had two single nucleotide polymorphisms distinguishing them from the remaining VGII strains. The VGI and VGIII alleles had 99% sequence identity, and these were 96% identical with the VGII alleles (data not shown).

Analysis of the *SXI1a* gene in the 108 MATa isolates revealed 11 allelic groups designated Ai to Av, Bi to Biv, C, and D. Phylogenetic analysis using PAUP* (39) found that the alleles clustered according to molecular type and sexual fecundity (Fig. 4). Two major clusters were apparent on the phylogram. Cluster 1 (groups Bi to Biv, C, and D) consisted of 20 isolates, including 19 VGII isolates and the single VGIII isolate. The latter was the only isolate in the divergent allelic group D. All isolates with the *SXI1a* B, C, or D alleles were fertile, and all the “robust maters” had a B or C allele. Cluster 2 (groups Ai to Av) included 88 isolates, of which 85 were infertile and 3 were weakly fertile (filaments and rare basidia without basidiospore chains).

The topology of the two clusters also differed. Cluster 2 had a very limited amount of sequence divergence, and each allele differed from the others by a single base substitution. In contrast, within cluster 1, the B alleles diverged from the consensus sequence by between 3.3% and 3.4% and by 2.3% and 3.7% in the C and D alleles, respectively.

The translated sequences for each *SXI1a* allele were aligned and compared with the predicted homeodomain region of the Sxi1α *C. neoformans* protein (16). In this region consisting of 62 amino acids, 18 substitutions occurred between the *C. neoformans* consensus sequence and the *C. gattii* alleles (71% sequence identity) (Table 5). One substitution (glutamine [K] to lysine [Q]) occurred at a predicted DNA-binding contact site, residue 44. The B and C alleles, despite being divergent in DNA sequence, had identical protein sequences (Fig. 4). Five substitutions distinguished the A, B, and C allelic groups. The
D allele shared three polymorphic residues with the A alleles and two with the B/C alleles and had two unique substitutions.

The $SXI2a$ sequence, though relatively large (2,529 bp), contained only three single nucleotide polymorphisms. One allele was found in two isolates, the second was found in a single isolate, and the remaining allele occurred in four isolates, which grouped with the $MATa$ consensus strain (E566) (Table 2).

**DISCUSSION**

Some Australian $C. gattii$ serotype B isolates retain sexual fecundity. Australia has a higher-than-average incidence of cryptococcosis caused by $C. gattii$, particularly in the indigenous population (4). However, to date, only three fertile clinical isolates have been identified, and all environmental isolates analyzed have appeared sterile (9). Epidemiological data could not eliminate the possibility that the previously identified fertile clinical isolates were from patients with reactivated infections in which the original disease was acquired elsewhere in the world. Therefore, these isolates were not included as Australian strains. In this study, we screened a collection of Australian environmental, clinical, and veterinary isolates and have identified 26 fertile strains capable of mating, including examples from each of the three different sources.

The sexual cycle of serotype D $C. neoformans$ var. neoformans.
has been studied extensively, and more recently, the sexual cycle of \textit{C. neoformans} var. \textit{gattii} was established (31). Mating in \textit{C. gattii} has been more of an enigma (9, 14, 24, 28). The sexual cycle of this species was recently recapitulated for the first time more than 25 years after it was first reported (21). This analysis involved screening of a collection of serotypes B and C clinical and environmental isolates and was facilitated by the discovery of a pair of serotype C strains that mate robustly with each other and with a large number of clinical and environmental isolates (9). Mating in previous studies and the current study remains limited to serotype C crossed with C or serotype C crossed with B (9, 21). Mating between two serotype B isolates has never been reported, which may be largely attributed to the rarity of mating-competent MATa strains. The single fertile MATa isolate identified in this study did not mate robustly and failed to mate with any of the 25 fertile MATa isolates. However, the finding of both mating competence and recombination in Australian VGII isolates in this and the accompanying study (2a) suggests that VGII MATa isolates are likely to occur in the Australian environment. We will now direct our search to regions where the VGII molecular type is endemic to identify fertile \textit{C. gattii} MATa strains.

The development of mating structures in response to coinulation with both serotypes D and C tester strains varied. Not all strains responded to the same testers, and the majority of isolates did not respond to any tester strain. It is therefore possible that given appropriate testers and mating conditions, more strains may emerge as fertile. However, data from this study are strongly supported by the observation of both recombination and clonality in a study of \textit{C. gattii} populations from Australia (2a). Comparisons of isolates common to both studies revealed that isolates belonging to recombining populations were almost all fertile. In contrast, all isolates analyzed that belonged to clonal populations were infertile or only very weakly fertile. This is one of the first studies that directly correlates the indirect assessment of both recombination and clonal propagation via multilocus genotype analysis with biological data on mating in a pathogenic fungus. A recent study by Litvintseva et al. identified a similar pattern of recombination and clonal expansion in \textit{C. neoformans} var. \textit{gattii} populations from Botswana. This study identified 14 isolates as recombining via amplified fragment length polymorphism analysis that also proved fertile and capable of producing recombinant progeny (27).

\textbf{Fertility in \textit{C. gattii} is strongly associated with molecular type.} Extensive studies have divided the three varieties of \textit{Cryptococcus} into molecular types based on differences in fingerprint patterns generated using a variety of molecular techniques (1, 29, 30). The four molecular types identified in \textit{C. gattii} exhibit an uneven global distribution, and in Australia, VGII predominates in both clinical and environmental collections. In the northern regions of Australia, clinical isolates have frequently been found to belong to molecular type VGII (35). This bias is reflected in the current study, in which the majority of clinical isolates were sourced from the Northern Territory. In contrast, the veterinary and environmental isolates were generally isolated from the southern states and were mostly VGII. A few VGIII isolates have been cultured from native trees in northern Australia; however, the exact environmental niche of this genotype remains unknown (5).

The majority of isolates capable of producing mating structures and all of the “robust maters” were found to be the VGII molecular type. This bias towards fertility is consistent with findings for an “outbreak” of cryptococcosis on Vancouver Island, Canada, where VGII isolates were also found to be highly fertile (9, 18). In contrast, 95% of VGII isolates were infertile. Interestingly, the single VGIII isolate in the study was weakly fertile, and considering that the two serotype C robustly mating tester strains also belong to molecular type VGIII, it would be interesting to analyze more VGIII strains to determine if this molecular type is also largely fertile. It is apparent that a link exists between VGII molecular type and fertility. A detailed analysis of the genetic variation between molecular types is required to determine the extent of this link, what genetic alterations might lead to infertility, and what the implications are for the future value of the serotype \textit{B} \textit{C. gattii} genome, which is of an apparently infertile VGI Australian isolate (WM276).

\textbf{Loss of fertility of Australian \textit{C. gattii} isolates.} Various degrees of mating ability, from robust mating with profuse basidiospore production to the extension of a few dikaryotic filaments to no mating, were observed. Fraser et al. (9) also

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{SXI1α allelic group} & \multicolumn{16}{|c|}{\textbf{Substitution at polymorphic amino acid site}} \\
\hline
& -1 & 1 & 2 & 7 & 9 & 10 & 11 & 15 & 22 & 28 & 32 & 33 & 37 & 39 & 40 & 43 & 44 & 45 \\
\hline
\textbf{CN (consensus)} & F & D & N & S & P & P & I & V & D & A & D & F & T & G & I & S & Q & V \\
\textbf{Ai (consensus)} & L & H & I & L & S & A & P & I & N & A & D & S & Y & G & M & G & K & V \\
\hline
\textbf{Ai1} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\textbf{Ai3} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\textbf{Ai4} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\textbf{Av} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\textbf{Avi} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\textbf{Bi} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\textbf{Bii} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\textbf{Biv} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\textbf{C} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\textbf{D} & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - & - \\
\hline
\end{tabular}
\caption{Distribution of amino acid substitutions within the \textit{SXI1α} predicted homeodomain region$^a$}
\footnotesize{$^a$ Residue 44, DNA-binding contact site (total of seven sites across region); CN, \textit{C. neoformans} consensus sequence (JEC21); Ai, \textit{C. gattii} (Ai) consensus sequence (WM276). Dots indicate that the residue corresponds to the Ai (consensus) residue.}
\end{table}
reported this range of mating response and proposed three possible mechanisms to explain the apparent loss of fertility in many C. gattii isolates. First, genetic barriers preventing mating between isolates of the same phenotypic species may lead to cryptic speciation. It is certainly possible that each of the VG types represents a separate cryptic species within C. gattii, as indicated by the marked genetic differentiation between each VG type when analyzed by DNA fingerprinting, sequencing, RFLP, or amplified fragment length polymorphism (2a, 17, 29, 30). In addition, complete genealogical congruence was seen with the URA5 and SXXIa gene trees, with no evidence of inter-VG hybridization (Fig. 4). However, VGII and VGIII isolates can clearly intermate, as the two serotype C tester strains were VGIII. To test for fertility within VGI, each of the weakly fertile MATα and MATα strains was paired with isolates of opposite mating type, but no evidence of mating was seen. Finally, in the companion study, all isolates that appeared infertile via mating analysis also appeared clonal via population genetic analysis, which strongly suggests that these isolates are truly infertile.

The second mechanism that may explain the preponderance of isolates that do not mate is a loss of sexual ability. Strains of C. neoformans have been found to spontaneously lose sexual ability when cultured for extended periods in the laboratory (48). After approximately 600 mitotic divisions on rich laboratory media, congenic MATα and MATα strains have been observed to lose mating and filamentation ability by more than 67% and 24%, respectively, without any significant loss in the vegetative growth rate of the cells. The vastly clonal population structure of C. gattii in the Australian environment might indicate that, similar to prolonged propagation on laboratory media, the eucalyptus tree might be conducive to asexual propagation and the eventual loss of sexual fertility over time.

Finally, Fraser and colleagues suggested that genetic alterations within the MAT locus might occur to render it nonfunctional. Sequence analysis of the SXXIa gene found a general partitioning of fertility with allelic groups, but this was confounded by cosegregation according to molecular type (Fig. 4). The translated amino acid sequence revealed a number of substitutions (Table 5). This region contains residues important for base contacts during DNA binding, and all such sites were conserved across the two Cryptococcus species with one exception, glutamine 44, for which all C. gattii alleles had a lysine residue. This is a nonconservative substitution at a critical functional residue and could alter the activity of the protein.

The MAT locus is very large (~100 kb) and contains at least 20 genes that are essential for mating function. It is therefore possible that other mutations that reduce or abolish fertility exist. As this locus does not recombine, polymorphisms within SXXIa might be linked to loss of function elsewhere in the locus. However, the presence of three weakly fertile VGI isolates within the ‘infertile’ cluster 2 (Fig. 4) indicates that SXXIa does not partition strictly according to fertility. Analysis of the MAT locus of fertile and infertile strains is currently under way to determine whether alterations might occur that make this region nonfunctional.

**What is the infectious propagule of infertile C. gattii strains?**

In the yeast Cryptococcus, the sexual cycle is important for the development of the basidiospore, which is thought to be the infectious propagule (3, 6). Basidiospores are small enough to penetrate the alveoli of the lung, <2 μm, are easily aerosolized, and have been shown to be infectious in murine models (38, 45, 50). In C. neoformans, basidiospores can also be produced in the absence of sexual recombination via monokaryotic fruiting (46). However, the vast majority of C. gattii strains cannot be induced to fruit, and in the few cases where some fruiting has been reported, this was limited to filament formation without the production of basidiospores (46, 49). In all the Australian C. gattii isolates analyzed here, monokaryotic fruiting was absent, suggesting that this method of spore production may not occur in Australian C. gattii or that the conditions used were not conducive to fruiting in these strains (26). Desiccated yeast cells have been suggested as an alternative infectious propagule to the basidiospore; however, there is evidence in C. neoformans that yeast cells are less infective and rapidly lose viability upon desiccation (38, 45). It would be interesting to analyze the infectivity of desiccated yeast cells of the fertile Australian VGI isolates to determine if these behave in a manner similar to that of desiccated C. neoformans cells.

The endemic nature of cryptococcosis due to C. gattii in some regions of Australia suggests that the infectious propagules, whether basidiospores or desiccated yeast cells, must be present in the environment, driving investigation into both the ecology and population structure of the fungus in this country. The identification of the two recombining VGI populations (2a) which also show evidence of fertility provides strong support that the mating observed under laboratory conditions is actually occurring in the natural environment in Australia. However, it appears that the majority of Australian isolates, including many causing human and animal infection, are infertile. The exact nature of the propagule responsible both for disease and for dispersing the fungus in the environment remains enigmatic.

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**REFERENCES**

1. Ali, S., C. R. Muller, and J. T. Epplen. 1986. DNA fingerprinting by oligonucleotide probes specific for simple repeats. Hum. Genet. 74:239–243.
2. Butlin, R. 2002. The costs and benefits of sex: new insights from old asexual lineages. Nat. Rev. Genet. 3:311–317.
2a. Campbell, L. T., B. J. Currie, M. Krockenberger, R. Malik, W. Meyer, J. Heitman, and D. Carter. 2005. Clonality and recombination in genetically differentiated subgroups of Cryptococcus gattii. 4:1403–1409.
3. Casadevall, A., and J. R. Perfect. 1998. Cryptococcus neoformans, 1st ed. American Society for Microbiology, Washington, D.C.
4. Chen, S., T. Sorrell, G. Nimmo, B. Speed, B. Currie, D. Ellis, D. Marriot, T. Pfeiffer, D. Parr, and K. Byth. 2000. Epidemiology and host- and variety-dependent characteristics of infection due to Cryptococcus neoformans in Australia and New Zealand. Clin. Infect. Dis. 31:499–508.
5. Chen, S. C., B. J. Currie, H. M. Campbell, D. A. Fisher, T. J. Pfeiffer, D. H. Ellis, and T. C. Sorrell. 1997. Cryptococcus neoformans var. gattii infection in northern Australia: existence of an environmental source other than known host eucalypts. Trans. R. Soc. Trop. Med. Hyg. 91:547–550.
6. Cohen, J., J. R. Perfect, and D. T. Durack. 1982. Cryptococcosis and the basidiospore. Lancet 1:301.
26. Lengeler, K. B., D. S. Fox, J. A. Fraser, A. Allen, K. Forrester, F. S. Deitrich, 28. Kwon-Chung, K. J., B. L. Wickes, L. Stockman, G. D. Roberts, D. Ellis, and 27. Hull, C. M., R. C. Davidson, and J. Heitman. 2003. Estimating the spontaneous mutation rate of loss of sex in the evolution of sex chromosomes. Eukaryot. Cell 2:704–718.

25. Kwon-Chung, K. J., B. L. Wickes, L. Stockman, G. D. Roberts, D. Ellis, and J. Heitman. 2003. A new species of Filobasidiella named Cryptococcus neoformans var. gattii strains from Australia. J. Clin. Microbiol. 41:2920–2926.

24. Hawkesworth, D. L., P. M. Kirk, B. C. Sutton, and D. N. Peggler. 2001. Ainsworth & Bisby's dictionary of the fungi, 8th ed. CAB International, Wallingford, U.K.

23. Hull, C. M., K. C. Davidson, and J. Heitman. 2002. Cell identity and sexual recombination in Cryptococcus neoformans are controlled by the mating-type-specific homedomain protein Su1A. Genes Dev. 16:3046–3060.

22. Kidd, S. E. 2003. Molecular epidemiology and characterisation of genetic structure to assess speciation within the Cryptococcus neoformans complex. Ph.D. thesis. University of Sydney, Sydney, Australia.

21. Kidd, S. E., F. Hagen, R. L. Tschanke, M. Huyhn, K. H. Bartlett, M. Fye, L. MacDougall, T. Boekhout, K. J. Kwon-Chung, and W. Meyer. 2004. A rare genotype of Cryptococcus gattii caused the cryptococcosis outbreak on Van-couver Island (British Columbia, Canada). Proc. Natl. Acad. Sci. USA 101:17258–17263.

20. Kurokawa, M. B., P. J. Canfield, and R. Malik. 2002. Cryptococcus neoformans in the koala (Phascolarctos cinereus): colonization by Cryptococcus neoformans var. gattii and investigation of environmental sources. Med. Mycol. 40:263–272.

19. Kwon-Chung, K. J. 1975. A new genus, Filobasidiella, the perfect state of Cryptococcus neoformans. Mycologia 67:1197–1200.

18. Kwon-Chung, K. J. 1976. A new species of Filobasidiella, the sexual state of Cryptococcus neoformans var. B and C serotypes. Mycologia 68:942–946.

17. Kwon-Chung, K. J., and J. E. Bennett. 1978. Distribution of α and α mating types of Cryptococcus neoformans among natural and clinical isolates. Am. J. Epidemiol. 108:337–340.

16. Kwon-Chung, K. J., T. Boekhout, J. W. Felé, and M. Diaz. 2002. Proposal to conserve the name Cryptococcus gattii against C. hondurianus and C. bacl- lapouras (Basidiomycota, Hymenomycetes, Tremellomycetidae). Taxon 51:804–806.

15. Kwon-Chung, K. J., B. L. Wickes, L. Stockman, G. D. Roberts, D. Ellis, and D. H. Howard. 1992. Virulence, serotype, and molecular characteristics of environmental isolates of Cryptococcus neoformans var. gattii. Infect. Immun. 60:1689–1754.

14. Kwon-Chung, K. J., J. E. Bennett (ed.). Medical mycology. Lea and Febiger, Philadelphia, Pa.

13. Kwon-Chung, K. J., B. L. Wickes, L. Stockman, G. D. Roberts, D. Ellis, and D. H. Howard. 1992. Virulence, serotype, and molecular characteristics of environmental isolates of Cryptococcus neoformans var. gattii. Infect. Immun. 60:1689–1754.

12. Kwon-Chung, K. J., J. E. Bennett (ed.). Medical mycology. Lea and Febiger, Philadelphia, Pa.

11. Kwon-Chung, K. J., B. L. Wickes, L. Stockman, G. D. Roberts, D. Ellis, and D. H. Howard. 1992. Virulence, serotype, and molecular characteristics of environmental isolates of Cryptococcus neoformans var. gattii. Infect. Immun. 60:1689–1754.

10. Kwon-Chung, K. J., J. E. Bennett (ed.). Medical mycology. Lea and Febiger, Philadelphia, Pa.

9. Kwon-Chung, K. J., B. L. Wickes, L. Stockman, G. D. Roberts, D. Ellis, and D. H. Howard. 1992. Virulence, serotype, and molecular characteristics of environmental isolates of Cryptococcus neoformans var. gattii. Infect. Immun. 60:1689–1754.

8. Kwon-Chung, K. J., B. L. Wickes, L. Stockman, G. D. Roberts, D. Ellis, and D. H. Howard. 1992. Virulence, serotype, and molecular characteristics of environmental isolates of Cryptococcus neoformans var. gattii. Infect. Immun. 60:1689–1754.

7. Ellis, D. H., and J. T. Pfeiffer. 1990. Natural habitat of Cryptococcus neoformans var. gattii. J. Clin. Microbiol. 28:1642–1644.

6. Fisher, R. A. 1930. The genetical theory of natural selection. Oxford University Press, Oxford, United Kingdom.

5. Fraser, J. A., R. L. Subaran, C. B. Nichols, and J. Heitman. 2003. Recapitulation of the sexual cycle of the primary fungal pathogen Cryptococcus neoformans var. gattii: implications for an outbreak on Vancouver Island, Canada. Eukaryot. Cell. Cell 2:704–718.

4. Fyfe, M., W. Black, M. Romney, P. Kibsey, L. MacDougall, M. Starr, M. Pearce, C. Stephen, L. Stein, S. Mak, B. Emerson, J. Isaac-Renton, and D. Patrick. 2002. Presented at the 5th International Conference on Cryptococ- cus and Cryptococcosis, Adelaide, Australia, 3 to 7 March 2002.

3. Goldard, M. R., H. Charles, J. Godfray, and A. Burt. 2005. Sex increases the efficacy of natural selection in experimental yeast populations. Nature 434:636–640.

2. Grigg, M. E., S. Bonnefoi, A. B. Hehl, Y. Suzuki, and J. C. Boothroyd. 2001. Suicide of male meiosis in Toxoplasma as the result of sexual recombination between two distinct anestries. Science 294:161–165.

1. Hallday, C. L., and D. A. Carter. 2003. Clonal reproduction and limited dispersal in an environmental population of Cryptococcus neoformans var. gattii. Proc. Natl. Acad. Sci. USA 100:17258–17263.