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# Association between fertility and molecular sub-type of global isolates of *Cryptococcus gattii* molecular type VGII

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The basidiomycetous yeast *Cryptococcus gattii,* is a primary pathogen which causes disease in apparently healthy humans and a wide range of animals. Recently, an outbreak of cryptococcosis caused by a previously uncommon genotype of *C. gattii,* VGII, emerged on Vancouver Island, British Columbia, Canada. Two pathogenic sub-types of VGII (designated VGIIa and VGIIb) were identified among these isolates. All of the isolates proved to be mating type α and had exceptionally high sporulation capacity. The common subtype, VGIIa, was more virulent than VGIIb in mice, suggesting a linkage between subtype and fertility/virulence. To test this hypothesis, we compared the fertility of 91 isolates from the Vancouver Island outbreak with that of 72 VGII isolates selected globally. Of all isolates, 69.94% were found to be fertile and exhibited clamp connections and basidiospores. The Vancouver isolates showed a high fertility rate of 84.2% as compared to only 29% of the 21 Australian isolates investigated. Mating type α strains were more fertile (72.79%) than mating type **a** (43.75%) ($p < 0.022$). Amongst the two subtypes of VGII a much higher proportion of VGIIa (91.7%) than VGIIb (33.3%) was fertile ($p < 0.001$). These results suggest that there is a clear correlation between the VGII subtypes of *C. gattii* and their mating/fertility. Further *in vitro* and *in vivo* investigations of more strains and congenic pairs are warranted.

**Keywords** fertility, mating, *Cryptococcus gattii*, VGII

## Introduction

Members of the *Cryptococcus* species complex, i.e., *C. neoformans* and *C. gattii*, are basidiomycetous yeasts that infect a wide range of animals and humans. *C. neoformans* is a global pathogen mainly associated with immunocompromised hosts, whereas *C. gattii* causes disease in apparently healthy hosts living in geographically restricted locations [1–4]. *C. neoformans* contains the following two varieties and three serotypes; *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and a hybrid (serotype AD). *C. gattii* is comprised of two serotypes, serotype B and C [1,3]. The *Cryptococcus* species complex has been divided into 8 major molecular types by M13 fingerprinting, *URA5*-RFLP and AFLP analysis [5–7]. These include VNI (=AFLP1) and VNII (=AFLP1A) (*C. neoformans* var. *grubii,* serotype A), VNIII (=AFLP3) (AD hybrid), VNIV (=AFLP2) (*C. neoformans* var. *neoformans*, serotype D), and VGI (=AFLP4), VGII (=AFLP6), VGIII (=AFLP5) and VGIV (=AFLP7) (*C. gattii,* serotypes B and C).

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Infection with the molecular type VGII was reported a decade ago in Australia to occur in animals and some humans [8,9]. Infections due to this molecular type were much less common than those caused by molecular type VGI. The latter accounted for nearly all cases of human cryptococcosis due to *C. gattii* [9, Meyer W, unpublished data]. Prior to 1999, *C. gattii* infections were reported mainly from tropical and subtropical regions [10,11]. The recent emergence of cryptococcosis due to the previously uncommon molecular type VGII amongst humans and animals in a temperate climatic zone on Vancouver Island, British Columbia, Canada is therefore of great interest. In particular, the incidence of human infection in this outbreak was substantially higher than that in endemic locations elsewhere in the world [12,13]. Since high concentration of cryptococci were found in the air on Vancouver Island, and since cryptococcosis is believed to develop following inhalation of infectious propagules from the environment, heavy exposure to environmental cryptococci was initially postulated to explain the increased virulence of the strains involved in the outbreak.

Subsequently, isolates from the outbreak were found to have an exceptionally high capacity to sporulate. Two independent mating studies consistently revealed high fertility rates (90%) amongst these isolates [13,14]. One subtype, VGIIa, identified by M13 fingerprinting and AFLP analysis, was much more common than subtype VGIIb and was more virulent in mice [15]. A link between the α mating type of *C. neoformans* var. *neoformans* and virulence in certain genetic backgrounds has been known for many years [16,17]. More recently it was shown that the α mating type of *C. neoformans* was more successful than the ***a*** mating type in crossing the blood brain barrier [18].

The aim of this study was to examine the relationship between virulence and fertility/mating ability in *C. gattii* by comparing the fertility of the Vancouver Island outbreak strains with that of globally collected VGII strains.

## Materials and methods

### *Strains and media*

One hundred and sixty three isolates of *C. gattii* molecular type VGII were retrieved from the following culture collections: Molecular Mycology Research Laboratory, Westmead Hospital, University of Sydney, Westmead, NSW, Australia; the Molecular Mycology and Mycobacteriology Laboratory, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand; and the Mycology Laboratory, National Institute of Health, Nonthaburi, Thailand (Table 1). All strains were grown on Saboraud Dextrose Agar (SDA; 2% peptone, 2% dextrose and 2% agar) for 48 h prior to sub-culture for mating experiments and for DNA extraction.

### *Reference strains*

A set of standard *C. neoformans* and *C. gattii* reference strains representing each of the eight major molecular types was also used in the investigations. These included; WM 148 (serotype A, VNI), WM 626 (serotype A, VNII), WM 628 (serotype AD, VNIII), WM629 (serotype D, VNIV), WM 179 (serotype B, VGI), WM 178 (serotype B, VGII), WM 175 (serotype B, VGIII), and WM 779 (serotype C, VGIV) [5]. Reference tester strains used in the mating experiments included; *C. gattii* clinical serotype C strains NIH312 (α mating type) [19] and B4546 (***a*** mating type) [20], and *crg1*α mutant derivatives (supermater tester strains), JF101 (α mating type) and JF109 (***a*** mating type) [14].

### *Verification of molecular type*

Fungal cells were disrupted by grinding in liquid nitrogen. DNA was isolated and purified as published previously [21]. The molecular type VGII was identified by *URA5* gene RFLP analysis using a double digest with the enzymes *Sau*96I and *Hha*I [5] and the molecular subtypes VGIIa and VGIIb were identified as described previously [13,15,22].

### *Mating type determination*

Two pairs of mating-type specific primers were used. MFαU (5′-TTCACTGCCATCTTCACCACC-3′) and MFαL (5′-TCTAGGCGATGACACAAAGGG-3′) primers were used to determine the α mating type [23]. MFa2U (5′-ACACCGCCTGTTACAATGGAC-3′) and MFa2L (5′-CAGCGTTTGAAGATGGACTTT-3′) were used to defined the ***a*** mating type [14]. PCR reactions and amplifications were performed as previously published [14,23].

### *Mating*

Reference tester strains for mating (serotype C NIH 312 and B-4546 [19,20]) and the supermater tester strains (serotype C *crg1Δ* mutants JF101 and JF109 [14]) were co-cultured with VGII strains of the opposite mating type. Cells from each mating partner were mixed on V8 juice agar (5% [vol/vol], 3mM $KH_2PO_4$, 4% [wt/vol] agar, adjusted to pH 5 [24]) and incubated

**Table 1** Details of *Cryptococcus gattii* molecular type VGII strains used in this study.

| Name | Mating type | Country | Source | VGII sub-molecular type | Fertility with mating reference tester strain[§] | Fertility with supermater tester strain[◆] | Reference |
|---|---|---|---|---|---|---|---|
| WM 178 | alpha | Australia | Clinical | N/A | F | F | [5] |
| ATCC 32608 | alpha | USA | Clinical | N/A | F | F | [9] |
| NIH 444 | alpha | USA | Clinical | a# | F | F | [39] |
| MCS115 | alpha | Thailand | Clinical | b** | F | F | [40] |
| MC-S-239 | alpha | Thailand | Clinical | N/A | F | F | [40] |
| MC-S-265 | alpha | Thailand | Clinical | N/A | F | F | [40] |
| New8 | alpha | Australia | Clinical | N/A | N | N | [12] |
| Warryndum | alpha | Australia | Clinical | N/A | N | N | [12] |
| NT-14 | alpha | Australia | Clinical | N/A | F | F | [12] |
| RAM 005 | alpha | Australia | Environmental | a** | N | N | [22] |
| RAM 002 | alpha | Australia | Environmental | N/A | N | N | [12] |
| RAM15 | alpha | Australia | Environmental | N/A | N | N | [12] |
| RDH-06 | alpha | Australia | Clinical | N/A | N | N | [12] |
| VPB 571-058 | alpha | Australia | Veterinary | N/A | N | N | [8] |
| VPB 571-073 | alpha | Australia | Veterinary | N/A | F | F | [8] |
| W12 | alpha | Australia | Clinical | N/A | N | N | [9] |
| ARN 001 | alpha | Australia | Environmental | N/A | N | N | [12] |
| LA 43 | alpha | Uruguay | Environmental | N/A | F | F | [5] |
| LA 44 | alpha | Brazil | Environmental | N/A | F | F | [5] |
| LA 45 | alpha | Brazil | Environmental | N/A | F | F | [5] |
| LA 47 | a | Brazil | Environmental | N/A | F | F | [5] |
| LA 55 | a | Brazil | Clinical | N/A | F | F | [5] |
| LA 57 | alpha | Brazil | Clinical | N/A | N | N | [5] |
| LA 61 | alpha | Brazil | Clinical | N/A | N | N | [5] |
| LA 84 | alpha | Brazil | Clinical | N/A | N | N | [5] |
| LA 150 | alpha | Brazil | Clinical | N/A | F | F | [5] |
| LA 151 | alpha | Brazil | Clinical | N/A | N | N | [5] |
| LA 295 | alpha | Argentina | Clinical | N/A | F | F | [5] |
| LA 337 | alpha | Brazil | Clinical | N/A | F | F | [5] |
| LA 338 | alpha | Brazil | Clinical | N/A | F | F | [5] |
| LA 362 | alpha | Brazil | Environmental | N/A | F | F | [5] |
| LA 381 | a | Venezuela | Clinical | N/A | N | N | [5] |
| LA 386 | a | Venezuela | Clinical | N/A | N | N | [5] |
| LA 387 | alpha | Venezuela | Clinical | N/A | F | F | [5] |
| LA 461 | a | Columbia | Clinical | N/A | N | N | [5] |
| LA 499 | a | Columbia | Clinical | N/A | N | N | [5] |
| LA 516 | a | Columbia | Clinical | N/A | N | N | [5] |
| LA 517 | a | Columbia | Clinical | N/A | N | N | [5] |
| LA 522 | alpha | Columbia | Clinical | N/A | N | N | [5] |
| LA 524 | a | Columbia | Clinical | N/A | N | N | [5] |
| LA 528 | alpha | Columbia | Clinical | N/A | F | F | [5] |
| LA 540 | a | Columbia | Clinical | N/A | F | F | [5] |
| LA 543 | a | Columbia | Clinical | N/A | N | N | [5] |
| LA 547 | a | Columbia | Clinical | N/A | N | N | [5] |
| LA 567 | a | Columbia | Clinical | N/A | N | N | [5] |
| LA 584 | a | Columbia | Clinical | N/A | F | F | [5] |
| LA 590 | alpha | Columbia | Clinical | N/A | N | N | [5] |
| LA 599 | a | Columbia | Clinical | N/A | N | N | [5] |
| CDC R268 | alpha | Canada | Clinical | a* | N | N | [13] |
| CDC R265 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R269 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R271 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R272 | alpha | Canada | Clinical | b* | F | F | [13] |
| CDC R273 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R322 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R360 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R368 | alpha | Canada | Clinical | a* | F | F | [13] |

**Table 1** (*Continued*)

| Name | Mating type | Country | Source | VGII sub-molecular type | Fertility with mating reference tester strain[§] | Fertility with supermater tester strain[◆] | Reference |
|---|---|---|---|---|---|---|---|
| CDC R369 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R406 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R409 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC AOR432 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R498 | alpha | Canada | Veterinary | a* | F | F | [13] |
| CDC R500 | alpha | Canada | Veterinary | a* | F | F | [13] |
| CDC R507 | alpha | Canada | Clinical | a* | F | F | [13] |
| CDC R540 | alpha | Canada | Clinical | a* | F | F | [13] |
| B 4534 | alpha | USA | Clinical | N/A | F | F | This study |
| R 634 | alpha | Canada | Clinical | a* | F | F | [13] |
| F 2596 | alpha | Canada | Veterinary | a* | F | F | [13] |
| F 2866 | alpha | Canada | Veterinary | a* | F | F | [13] |
| F 2932 | alpha | Canada | Clinical | a* | F | F | [13] |
| F 3016 | alpha | Canada | Veterinary | a* | F | F | [13] |
| F 3179 | alpha | Canada | Clinical | a* | F | F | [13] |
| F 3197 | alpha | Canada | Clinical | a* | N | N | [13] |
| E 113 | alpha | Canada | Environmental | a* | F | F | [13] |
| 99 MR10 | alpha | Canada | Clinical | a* | F | F | [13] |
| E 123 | alpha | Canada | Clinical | a* | F | F | [13] |
| ENV 133 | alpha | Canada | Environmental | b* | N | F | [13] |
| ENV 153 | alpha | Canada | Environmental | a* | F | F | [13] |
| ENV 124 | alpha | Canada | Environmental | a* | F | F | [13] |
| ENV 130 | alpha | Canada | Environmental | a* | F | F | [13] |
| ENV 125 | alpha | Canada | Environmental | a* | F | F | [13] |
| ENV 131 | alpha | Canada | Environmental | a* | F | F | [13] |
| ENV 129 | alpha | Canada | Environmental | b* | N | F | [13] |
| ENV 152 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB1 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB2 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB3 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB4 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB5 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB8 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB9 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB11 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB13 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB14 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB15 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB17 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB19 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB20 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB22 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB25 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB26 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB28 | alpha | Canada | Environmental | b* | N | F | [13] |
| RB29 | alpha | Canada | Environmental | a* | N | N | [13] |
| RB30 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB31 | alpha | Canada | Environmental | b* | N | F | [13] |
| RB32 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB33 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB34 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB35 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB37 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB39 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB40 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB45 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB46 | alpha | Canada | Environmental | a* | F | F | [13] |

**Table 1** (*Continued*)

| Name | Mating type | Country | Source | VGII sub-molecular type | Fertility with mating reference tester strain[§] | Fertility with supermater tester strain[◆] | Reference |
|---|---|---|---|---|---|---|---|
| RB48 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB50 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB52 | alpha | Canada | Environmental | b* | F | F | [13] |
| RB54 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB55 | alpha | Canada | Environmental | a* | N | N | [13] |
| RB56 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB57 | alpha | Canada | Environmental | b* | F | F | [13] |
| RB58 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB59 | alpha | Canada | Environmental | a* | F | F | [13] |
| 113A-5 | alpha | Canada | Environmental | a* | F | F | [13] |
| 129A-1 | alpha | Canada | Environmental | a* | F | F | [13] |
| 152A-1 | alpha | Canada | Environmental | a* | F | F | [13] |
| 152A-2 | alpha | Canada | Environmental | a* | F | F | [13] |
| 152A-3 | alpha | Canada | Environmental | a* | F | F | [13] |
| 152A-4 | alpha | Canada | Environmental | a* | F | F | [13] |
| 152A-5 | alpha | Canada | Environmental | a* | F | F | [13] |
| 152A-6 | alpha | Canada | Environmental | b* | F | F | [13] |
| MAC9 | alpha | Canada | Environmental | a* | N | N | [13] |
| RB61 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB62 | alpha | Canada | Environmental | a* | N | N | [13] |
| RB63 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB67 | alpha | Canada | Environmental | b* | N | F | [13] |
| RB69 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB90 | alpha | Canada | Environmental | a* | N | N | [13] |
| RB96 | alpha | Canada | Environmental | a* | F | F | [13] |
| RB42 | alpha | Canada | Environmental | a* | F | F | [13] |
| CBS7750 | alpha | USA | Environmental | a** | F | F | [22] |
| GC 27 | alpha | Australia | Environmental | N/A | N | N | [12] |
| NT -07 | alpha | Australia | Clinical | N/A | N | N | [12] |
| WM1008 | alpha | Australia | Environmental | b** | F | F | [22] |
| Bandiaga | alpha | Australia | Environmental | N/A | N | N | [12] |
| MA 21832 | alpha | Australia | Veterinary | N/A | F | F | [8] |
| 571116 | alpha | Australia | Veterinary | N/A | N | F | [8] |
| 1607/96 | alpha | Australia | Veterinary | N/A | N | N | [8] |
| TP 0494 | alpha | Australia | Environmental | N/A | N | N | [12] |
| McBride | alpha | Australia | Veterinary | N/A | F | F | [8] |
| DMST 20763 | alpha | Thailand | Clinical | N/A | F | F | [41] |
| DMST 20764 | alpha | Thailand | Clinical | N/A | F | F | [41] |
| DMST 20765 | alpha | Thailand | Clinical | N/A | F | F | [41] |
| DMST 20766 | alpha | Thailand | Clinical | N/A | F | F | [41] |
| DMST 20767 | alpha | Thailand | Clinical | N/A | F | F | [41] |
| DMST 20768 | alpha | Thailand | Clinical | N/A | N | F | [41] |
| 47-5061 | alpha | Thailand | Clinical | N/A | F | F | This study |
| 47-5055 | alpha | Thailand | Clinical | N/A | F | F | This study |
| 47-2158 | alpha | Thailand | Clinical | N/A | N | F | This study |
| 47-4995 | alpha | Thailand | Clinical | N/A | F | F | This study |
| AV54S | alpha | Greece | Clinical | N/A | F | F | [32] |
| AV54W | alpha | Greece | Clinical | N/A | F | F | [32] |
| AV55 | a | Greece | Clinical | N/A | F | F | [32] |

N/A = No data available or they are different sub-type from VGIIa and VGIIb, N = non-fertile; F = fertile; [§] = Mating reference tester strains are NIH312 ($\alpha$ mating type) and B4546 (***a*** mating type); ◆ = Supermater tester strains are *crg1*$\alpha$ mutant derivatives, JF101 ($\alpha$ mating type) and JF109 (***a*** mating type); Sub-molecular types were defined according to the following publications: *[13]; **[22] and #[15].

in darkness for up to 4 weeks. Sexual reproduction was confirmed by the presence of basidiospore chains and fused clamp connections. The clamp connections were stained by Calcofluor white and visualized under a fluorescent microscope according to an established protocol [14]. All strains were individually incubated

on V8 juice agar as described above, to differentiate haploid fruiting from mating.

*Statistical analysis*

The software SPSS 15.0 was used for statistical analysis by Fisher's Exact test. Significance was defined as a $P$ value $<0.05$.

## Results

One hundred and sixty three isolates from 10 countries representing 5 continents were studied: 1 from Argentina, 21 from Australia, 12 from Brazil, 91 from Canada, 14 from Columbia, 3 from Greece, 13 from Thailand, 1 from Uruguay, 4 from the USA, and 3 from Venezuela. They were all tested for their ability to mate with standard strains of the opposite mating type (Table 1).

Strains of mating type **a** were present only among the South American isolates (51.61%) and those from Greece (a single isolate; Figs. 1 and 2). Of the total of 163 isolates, 69.94% were found to be fertile. Regional differences in fertility (Table 2) were significant ($P<0.001$). High fertility rates ($>75\%$) were observed in the tested Greek (3/3), US (3/4) and Canadian (77/91) strains. The $\alpha$ mating type strains were more fertile (72.79%) than the ***a*** mating type strains (43.75%) ($P<0.022$). Amongst the South American isolates, 62.5% (10/16) of the $\alpha$ mating type strains and 40% (6/15) of the ***a*** mating type strains were able to mate ($P<0.289$). A higher proportion of globally collected VGIIa strains (91.67%) than VGIIb strains (33.33%) mated ($P<0.001$) (Table 3). Fertility rates of isolates from every continent were consistently higher when tested with the supermater tester strains, JF101 ($\alpha$ mating type) and JF109 (***a*** mating type) [14]. All fertile isolates formed hyphae and produced typical bacillary forms of basidiospores and clamp connections (Fig. 3). No haploid fruiting was detected.

## Discussion

Human and animal exposure to environmental fungi, and hence infection, is proportional to the degree of dispersion of potential pathogens within the environment. The infectious propagule of *C. neoformans* is not known but it has been proposed that basidiospores or conidiospores may act in this capacity [25]. Since cryptococcosis is acquired by the respiratory route, the small size of basidiospores would facilitate entry into the lower respiratory tract. *Cryptococcus* has been reported to develop spore-like forms by either sexual reproduction or a non-sexual process known as haploid fruiting [26]. Thus, the role of the sexual cycle and the genes of the $\alpha$ and ***a*** mating type loci have attracted attention since they directly control both sexual and asexual sporulation.

An association between virulence and the $\alpha$ mating type of *C. neoformans* was initially suggested when a predominance of the $\alpha$ mating type was discovered in global cryptococcal populations [27]. The $\alpha$ mating type was then shown to be more virulent than the ***a*** mating type in certain genetic backgrounds [16,17] and it was better able to penetrate the blood brain barrier in a mice model [28]. A similar mating type bias has been reported in *C. gattii* strains from areas endemic for infections caused by this member of the genus, including Australia [29], Canada [13] and Brazil [30]. Our results extend these findings using a large global collection of 163 *C. gattii* VGII strains from several additional countries and regions. We also observed a predominance of the $\alpha$ mating type, except in South America, where there was an equal proportion of ***a*** and $\alpha$ mating types. There is a potential isolate selection

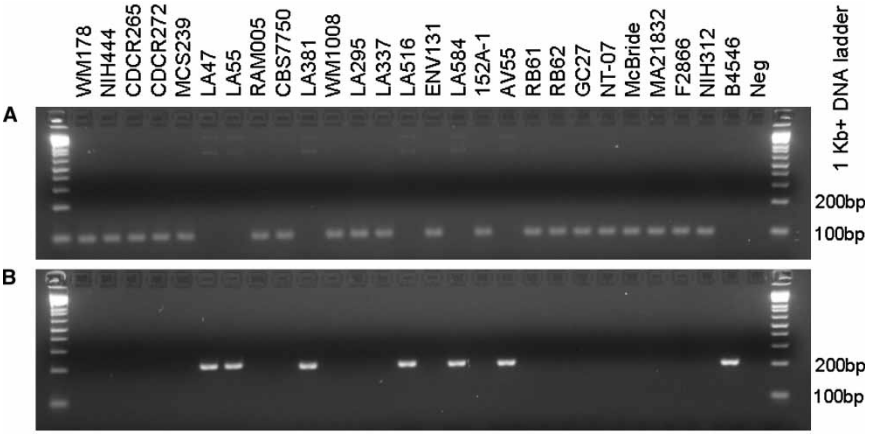


**Fig. 1** A specific PCR of A) mating type $\alpha$ with the primers MF$\alpha$U/MF$\alpha$L and B) mating type **a** with the primers MF**a**2U/MF**a**2L. Strains of mating type $\alpha$ and **a** gave 101bp and 213bp amplification products respectively compared with the controls: $\alpha$ = NIH312, **a** = B4546 and Neg = negative control. All were run against a 1 Kb plus a DNA marker (Invitrogen®).

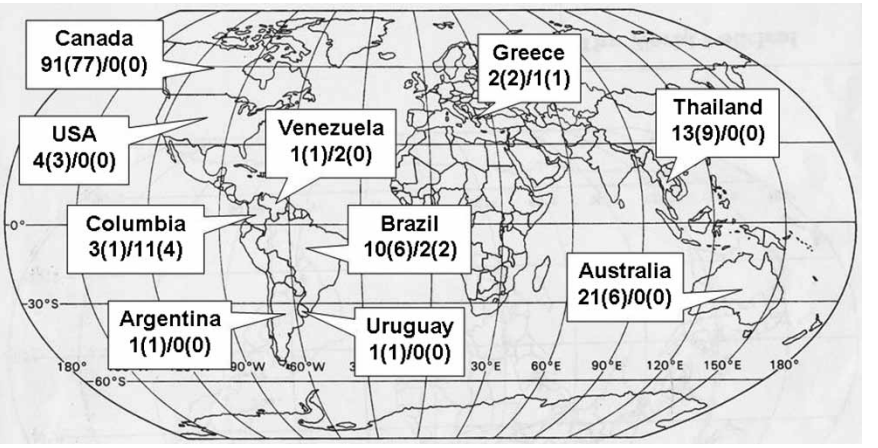


**Fig. 2** Mating type and fertility distribution of global VGII strains showing numbers of isolates collected from different countries (mating type α/mating type **a**). Numbers in the brackets represent number of fertile strains for each mating type.

**Table 2** Mating type and percentage of fertile isolates.

| Geographic region (Total # of strains) | Mating type (α/a) | Fertility with Mating reference tester strains* | Fertility with supermater tester strains◆ |
|---|---|---|---|
| Australia (21) | 21/0 | 28.57% | 33.33% |
| Canada (91) | 91/0 | 84.62% | 92.31% |
| Greece (3) | 2/1 | 100% | 100% |
| South America (31) | 16/15 | 51.61% | 64.52% |
| Thailand (13) | 13/0 | 69.23% | 100% |
| USA (4) | 4/0 | 75% | 100% |

Regional difference in fertility is statistically significant ($P < 0.001$); *Mating reference tester strains are NIH312 (α mating type) and B4546 (***a*** mating type); ◆Supermater tester strains are *crg1*α mutant derivatives, JF101 (α mating type) and JF109 (***a*** mating type).

bias in favor of ***a*** mating types in South American strains studied because our collection included isolates of *C. gattii* from Columbia where it was recently reported that there is a high proportion of ***a*** mating type strains [31]. However our finding of an equal proportion of ***a*** and α mating types among South American isolates strongly suggests that sexual recombination is occurring in this region of the world. An additional ***a*** mating type strain was unexpectedly recovered from Greece. Whether the *C. gattii* strain isolated in Greece was imported from elsewhere or originated in this temperate-climate zone remains controversial [32], but only a small number of isolates (3 isolates) were tested. Further environmental surveys are needed to resolve these questions.

**Table 3** Fertility of the isolates according to molecular subtype.

| Molecular subtype (total # of strains) | Fertile/Infertile (mating reference tester strains)* | Fertile/Infertile (supermater tester strains)◆ |
|---|---|---|
| VGIIa (84) | 77/7 | 77/7 |
| VGIIb (12) | 4/8 | 11/1 |

The VGIIa strains are more fertile than the VGIIb strains ($P < 0.001$); *Mating reference tester strains are NIH312 (α mating type) and B4546 (***a*** mating type); ◆Supermater tester strains are *crg1*α mutant derivatives, JF101 (α mating type) and JF109 (***a*** mating type).

The ability to produce spores is dependent on the fertility of each strain, which may in turn determine the virulence of the organism as found in *Toxoplasma gondiii* in which the ability to undergo sexual reproduction correlated with virulence [33,34]. There is also evidence from studies in *Saccharomyces cerevisiae* that the ability to undergo sexual recombination facilitates adaptation to new environments by increasing genetic diversity. Strains capable of sexual recombination were more tolerant to a new harsh environment [35,36]. Furthermore, links between mating and pathogenicity have been recognized for several fungi. In *Ustilago maydis*, a basidiomycous fungal pathogen of plants, pathogenesis and sexual development are intricately interconnected since disease is completely dependent on the ability of the fungus to accomplish a complete sexual cycle [37]. A recent study demonstrated that the a/α genotype of *Candida albicans* has a competitive advantage over a/a and α/α offspring in colonizing hosts [38]. Our findings support a direct relationship between fertility and virulence in *C. gattii*, since the more virulent subtype, VGIIa, was more fertile than the

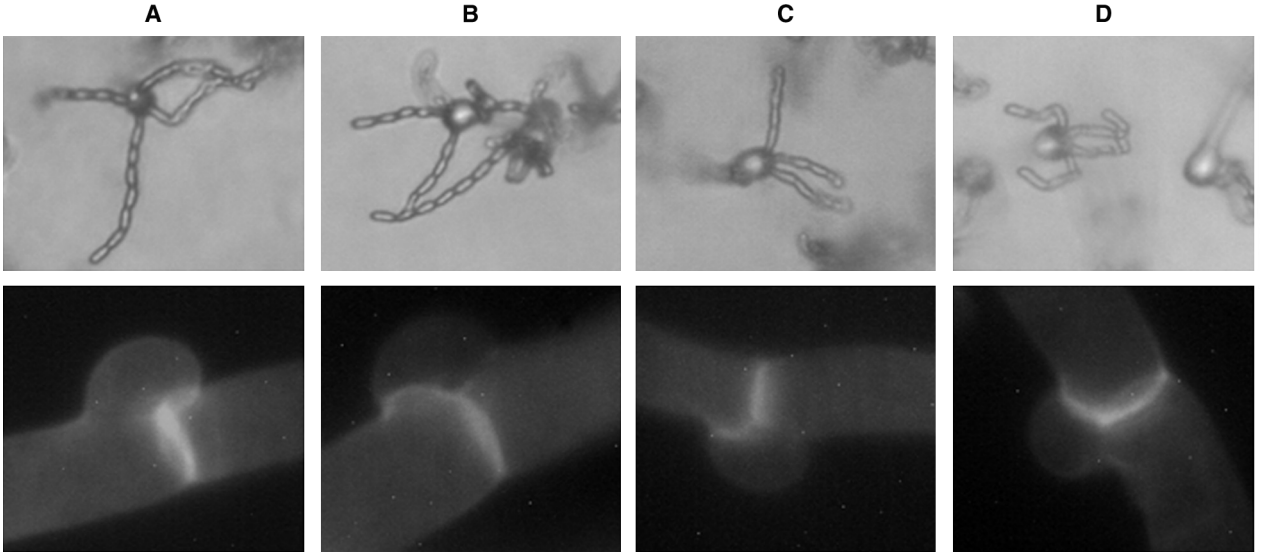


**Fig. 3** Mating of the *Cryptococcus gattii* isolates against the opposite standard mating type partners: (A) R265 (serotype B, VGIIa, mating type α) × B4546 (serotype C, VGIII, mating type **a**), (B) R272 (serotype B, VGIIb, mating type α) × B4546 (serotype C, VGIII, mating type **a**), (C) AV55 (serotype B, VGII, mating type **a**) × NIH312 (serotype C, VGIII, mating type α) and (D) LA47 (serotype B, VGII, mating type **a**) × NIH312 (serotype C, VGIII, mating type α). The top row shows basidiospore formation and the lower row shows clamp connections of each cross.

less virulent subtype, VGIIb. This is consistent with the fact that the incidence of cryptococcosis due to *C. gattii* VGIIa in Canada is much higher than reported elsewhere [13].

To confirm our findings, investigation of the virulence of larger numbers of strains of the two molecular subtypes VGIIa and VGIIb found on Vancouver Island and elsewhere are the focus of an ongoing international research effort. In addition, though fertility of the α mating type strains of *C. gattii* was clearly superior than that of the ***a*** mating type strains, further confirmation of this association should be derived from virulence testing of several congenic pairs of VGIIa and VGIIb.

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## References

1 Casadevall A, Perfect JR. *Cryptococcus neoformans*. Washington, DC: ASM Press; 1998.
2 Krockenberger MB, Canfield PJ, Malik R. *Cryptococcus neoformans* var. *gattii* in the koala (*Phascolarctos cinereus*): a review of 43 cases of cryptococcosis. *Med Mycol* 2003; **41**: 225–234.
3 Kwon-Chung KJ, Boekhout T, Fell JW, Diaz M. Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. basillisporus* (Basidiomycota, Hymenomycetes, Tremellomycetidae). *Taxon* 2002; **51**: 804–806.
4 Stephen C, Lester S, Black W, Fyfe M, Raverty S. Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. *Can Vet J* 2002; **43**: 792–794.
5 Meyer W, Castaneda A, Jackson S, Huynh M, Castaneda E. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis* 2003; **9**: 189–195.
6 Meyer W, Marszewska K, Amirmostofian M, *et al*. Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA-a pilot study to standardize techniques on which to base a detailed epidemiological survey. *Electrophoresis* 1999; **20**: 1790–1799.

7 Boekhout T, Theelen B, Diaz M, *et al*. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* 2001; **147**: 891–907.
8 Sorrell TC, Brownlee AG, Ruma P, *et al*. Natural environmental sources of *Cryptococcus neoformans* var. *gattii*. *J Clin Microbiol* 1996; **34**: 1261–1263.
9 Kwon-Chung KJ, Bennett JE. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. *Am J Epidemiol* 1984; **120**: 123–130.
10 Sorrell TC, Chen SC, Ruma P, *et al*. Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. *J Clin Microbiol* 1996; **34**: 1253–1260.
11 Sorrell TC. *Cryptococcus neoformans* variety *gattii*. *Med Mycol* 2001; **39**: 155–168.
12 Chen S, Sorrell T, Nimmo G, *et al*. Epidemiology and host- and variety-dependent characteristics of infection due to *Cryptococcus neoformans* in Australia and New Zealand. Australasian Cryptococcal Study Group. *Clin Infect Dis* 2000; **31**: 499–508.
13 Kidd SE, Hagen F, Tscharke RL, *et al*. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). *Proc Natl Acad Sci USA* 2004; **101**: 17258–17263.
14 Fraser JA, Subaran RL, Nichols CB, Heitman J. Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*: implications for an outbreak on Vancouver Island, Canada. *Eukaryot Cell* 2003; **2**: 1036–1045.
15 Fraser JA, Giles SS, Wenink EC, *et al*. Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature* 2005; **437**: 1360–1364.
16 Kwon-Chung KJ, Edman JC, Wickes BL. Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infect Immun* 1992; **60**: 602–605.
17 Nielsen K, Marra RE, Hagen F, *et al*. Interaction between genetic background and the mating-type locus in *Cryptococcus neoformans* virulence potential. *Genetics* 2005; **171**: 975–983.
18 Nielsen K, Cox GM, Litvintseva AP, *et al*. *Cryptococcus neoformans* alpha strains preferentially disseminate to the central nervous system during coinfection. *Infect Immun* 2005; **73**: 4922–4933.
19 Wickes BL, Moore TD, Kwon-Chung KJ. Comparison of the electrophoretic karyotypes and chromosomal location of ten genes in the two varieties of *Cryptococcus neoformans*. *Microbiology* 1994; **140**(Pt 3): 543–550.
20 Varma A, Kwon-Chung KJ. Construction of stable episomes in *Cryptococcus neoformans*. *Curr Genet* 1998; **34**: 60–66.
21 Meyer W, Lieckfeldt E, Kuhls K, *et al*. DNA- and PCR-fingerprinting in fungi. *Exs* 1993; **67**: 311–320.
22 Kidd SE, Guo H, Bartlett KH, Xu J, Kronstad JW. Comparative gene genealogies indicate that two clonal lineages of *Cryptococcus gattii* in British Columbia resemble strains from other geographical areas. *Eukaryot Cell* 2005; **4**: 1629–1638.
23 Halliday CL, Bui T, Krockenberger M, *et al*. Presence of alpha and a mating types in environmental and clinical collections of *Cryptococcus neoformans* var. *gattii* strains from Australia. *J Clin Microbiol* 1999; **37**: 2920–2926.
24 Kwon-Chung KJ, Bennett JE, Rhodes JC. Taxonomic studies on *Filobasidiella* species and their anamorphs. *Antonie Van Leeuwenhoek* 1982; **48**: 25–38.
25 Wickes BL. The role of mating type and morphology in *Cryptococcus neoformans* pathogenesis. *Int J Med Microbiol* 2002; **292**: 313–329.
26 Lin X, Heitman J. The biology of the *Cryptococcus neoformans* species complex. *Annu Rev Microbiol* 2006; **60**: 69–105.
27 McClelland CM, Chang YC, Varma A, Kwon-Chung KJ. Uniqueness of the mating system in *Cryptococcus neoformans*. *Trends Microbiol* 2004; **12**: 208–212.
28 Nielsen K, Cox GM, Wang P, *et al*. Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic a and alpha isolates. *Infect Immun* 2003; **71**: 4831–4841.
29 Campbell LT, Currie BJ, Krockenberger M, *et al*. Clonality and recombination in genetically differentiated subgroups of *Cryptococcus gattii*. *Eukaryot Cell* 2005; **4**: 1403–1409.
30 Trilles L, Lazera M, Wanke B, Theelen B, Boekhout T. Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Med Mycol* 2003; **41**: 383–390.
31 Escandon P, Sanchez A, Martinez M, Meyer W, Castaneda E. Molecular epidemiology of clinical and environmental isolates of the *Cryptococcus neoformans* species complex reveals a high genetic diversity and the presence of the molecular type VGII mating type a in Colombia. *FEMS Yeast Res* 2006; **6**: 625–635.
32 Velegraki A, Kiosses VG, Pitsouni H, *et al*. First report of *Cryptococcus neoformans* var. *gattii* serotype B from Greece. *Med Mycol* 2001; **39**: 419–422.
33 Su C, Evans D, Cole RH, *et al*. Recent expansion of *Toxoplasma* through enhanced oral transmission. *Science* 2003; **299**: 414–416.
34 Grigg ME, Bonnefoy S, Hehl AB, Suzuki Y, Boothroyd JC. Success and virulence in *Toxoplasma* as the result of sexual recombination between two distinct ancestries. *Science* 2001; **294**: 161–165.
35 Goddard MR, Godfray HC, Burt A. Sex increases the efficacy of natural selection in experimental yeast populations. *Nature* 2005; **434**: 636–640.
36 Grimberg B, Zeyl C. The effects of sex and mutation rate on adaptation in test tubes and to mouse hosts by *Saccharomyces cerevisiae*. *Evolution Int J Org Evolution* 2005; **59**: 431–438.
37 Banuett F. Genetics of *Ustilago maydis*, a fungal pathogen that induces tumors in maize. *Annu Rev Genet* 1995; **29**: 179–208.
38 Lockhart SR, Wu W, Radke JB, Zhao R, Soll DR. Increased virulence and competitive advantage of a/alpha over a/a or alpha/alpha offspring conserves the mating system of *Candida albicans*. *Genetics* 2005; **169**: 1883–1890.
39 Kwon-Chung KJ. A new species of *Filobasidiella*, the sexual state of *Cryptococcus neoformans* B and C serotypes. *Mycologia* 1976; **68**: 943–946.
40 Sukroongreung S, Nilakul C, Ruangsomboon O, Chuakul W, Eampokalap B. Serotypes of *Cryptococcus neoformans* isolated from patients prior to and during the AIDS era in Thailand. *Mycopathologia* 1996; **135**: 75–78.
41 Poonwan N, Mikami Y, Poosuwan S, *et al*. Serotyping of *Cryptococcus neoformans* strains isolated from clinical specimens in Thailand and their susceptibility to various antifungal agents. *Eur J Epidemiol* 1997; **13**: 335–340.