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Cryptococcus gattii, no longer an accidental pathogen?

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Abstract

Cryptococcus gattii is an environmentally occurring pathogen that is responsible for causing cryptococcosis marked by pneumonia and meningoencephalitis in humans and animals. *C. gattii* can form long-term associations with trees and soil resulting in the production of infectious propagules (spores and desiccated yeast). The ever expanding reports of clinical and environmental isolation of *C. gattii* in temperate climates strongly imply *C. gattii* occurs world-wide. The key ability of yeast and spores to enter, survive, multiply, and exit host cells and to infect immunocompetent hosts distinguishes *C. gattii* as a primary pathogen and suggest evolution of *C. gattii* pathogenesis as a result of interaction with plants and other organisms in its environmental niche. Here we summarize the historical literature on *C. gattii* and recent literature supporting the world-wide occurrence of the primary pathogen *C. gattii*.

Keywords

Cryptococcus; Cryptococcosis, meningitis; fungal infection; molecular typing; pneumonia; fungal virulence; VG groups; pathogenesis; emerging outbreak, same-sex mating; opposite sex mating; HIV/AIDS

Introduction

Cryptococcus gattii (formerly *C. neoformans* var. *gattii*) was first recognized by its unique cigar-shaped, elongated morphology (Figure 1) in the cerebral spinal fluid of an infected Congolese boy and later raised to species level due to differences from *C. neoformans* in morphology, biochemical attributes, and molecular sequences [1–5]. Cryptococcosis is a disease resulting from the inhalation of the infectious propagules of *Cryptococcus* from the environment. An initial lung infection can be rapidly cleared, progress to fulminant infection, or persist asymptomatically for long periods of time latently and then disseminate through the bloodstream into the brain, causing neurological symptoms, meningoencephalitis, and eventually death if untreated. Studies of virulence in *C. gattii* have benefitted from comparison with the well-studied sibling species, *Cryptococcus neoformans*. Many of the virulence factors of *C. neoformans* are conserved in *C. gattii*, including capsule, melanin, and the ability to grow at 37°C, the mechanisms of regulation are not always conserved [5–7].

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Historically, *C. gattii* was considered endemic in tropical and subtropical regions of the world. However, beginning in 1999, there has been a continuous and expanding outbreak in the temperate region in the Pacific Northwest. This outbreak was first recognized by the concurrent identification of humans and animals presenting with pneumonia and/or meningoencephalitis in 1999 on Vancouver Island [8, 9]. The infection rate of *C. gattii* on Vancouver Island between 1999 and 2003 was found to be substantially higher (~9 times) than in Australia, an endemic region for *C. gattii* [10]. The outbreak in the Pacific Northwest (PNW) was preceded many years earlier by a human infection with *C. gattii* (NIH444 =CBS6956/ATCC 32609), which was isolated in the 1970's from the sputum of a patient in Seattle, WA, U.S.A., which lies in close proximity to Vancouver Island, B.C., Canada, and shares a similar climate [5, 11]. Furthermore, prior to the recognition of the outbreak, molecular type VGII was isolated from the environment in the San Francisco area in 1990 (CBS7750) [12]. In 2004, the outbreak expanded beyond Vancouver Island, with cases observed on the Canadian mainland in humans who had not traveled to Vancouver Island or other locations known to be high risk for *C. gattii* [13]. The outbreak progressed, and starting in 2005/2006, human and animal cases began to be reported in Oregon and Washington [14]. It is currently unknown how far it will eventually spread, and what barriers may exist to further spread. Monitoring the progression of this outbreak is important for public health officials as well as physicians and veterinarians.

Virulence

Under laboratory conditions, both yeast cells and spores can initiate infection and cause disease in animal models [15, 16]. Due to the smaller size of spores they may be more effectively aerosolized in nature and more efficiently enter the bronchial tubes and alveolar spaces within the lungs [15, 17]. Additionally, spores are implicated in promoting infection and disease development because, in contrast to yeast cells, they do not require opsonization for phagocytosis by macrophages [16]. The ability to persist inside of host cells is a key virulence attribute. In an evolutionary sense, this is likely linked to the interaction between *Cryptococcus* species, its environmental niche, and other organisms (amoeba, nematodes, insects, plants, and other fungi) [18–23]. However, virulence in the human host entails survival and non-lytic expulsion after phagocytosis by macrophages (vomocytosis) [22, 24]. *Cryptococcus* is a facultative intracellular pathogen, and proliferation within macrophages and the formation of tubular mitochondria has been correlated with virulence in the murine and *Galleria* model systems [25, 26]. Increased intracellular proliferation rates (IPR) were associated with upregulation in protein degradation and synthesis, carbohydrate metabolism, stress response, nucleotide metabolism, vesicular fusion, and transport [25]. Fusion of mitochondria resulted in morphological changes and the appearance of tubular mitochondria, which may protect against cell death and mutations by allowing complementation between mitochondria. Macrophages are implicated in both the dissemination of *Cryptococcus* from the lungs and the ferrying of *Cryptococcus* across the blood-brain barrier to the central nervous system [27]. The ability of spores to be more readily phagocytosed may dramatically impact latency and the course of infection.

Molecular types

Historical perspectives on cryptococcosis due specifically to *C. gattii* in AIDS patients are restricted due to a previous lack of differentiating *C. gattii* from *C. neoformans*. Cryptococcal serotypes were first readily distinguished by agglutination assays into Serotypes A, B, C, and D and later serotypes were associated with *C. neoformans* (Serotype A, D, and AD hybrids) and *C. gattii* (Serotype B and C) [28–31]. Canavanine-glycine-bromothymol blue (CGB) agar is now widely used for initial identification of *C. gattii* from clinical samples [30, 32, 33]. Genotyping of *Cryptococcus* species has resulted in major

advances in differentiating *C. gattii* isolates. PCR fingerprinting and amplified fragment length polymorphisms (AFLP) have redefined our understanding of *C. gattii* into 4 molecular types: VGI, VGII, VGIII, and VGIV [31, 34–38]. *C. gattii-C. neoformans* hybrids are rare, obtained only from clinical samples, and identified as a result of Amplified fragment length polymorphism (AFLP), and multi-locus sequencing typing (MLST) analysis (AFLP8 and AFLP9) [39–41]. Only VGI and VGII have been associated thus far with forming hybrids with *C. neoformans* [40, 42].

C. gattii VGI is the most prevalent type of veterinary, clinical, and environmental isolate sampled world-wide. Clinical and veterinary cases associated with *C. gattii* VGI in the Netherlands, China, PNW, Eastern USA, combined with the recent documented environmental isolation of VGI from the Netherlands, Spain, Italy, and PNW strongly suggest a greatly expanded ecological niche of VGI outside the historically endemic regions [43–49]. VGII has a more restricted distribution and is most prominently associated with disease in the PNW and Australia although it has also been associated with disease in Central and South America, India, Korea, and China [50–55]. VGI and VGII primarily infect immunocompetent or non-AIDS immunocompromised hosts. *C. gattii* VGIII and VGIV are associated with immunocompromised AIDS patients but can also occur in patients with no apparent immune dysfunction [56–58]. The clinical presence of VGIII is prominent in California but recent reports from New Mexico suggest that VGIII has established an environmental niche in the Southwestern USA. In stark contrast to the clinical presence of VGI, VGII, and VGIII only two isolates of *C. gattii*, CBS7750 (VGII, 1990, San Francisco) and WM161 (VGIII, San Diego), have ever been recovered environmentally from California [31, 59]. In the Southwestern USA, identification of the ecological reservoir(s) of *C. gattii* appears elusive even with increased environmental sampling [50, 60, 61]. Furthermore VGIII has been associated with sporadic infections in Mexico, Colombia, Brazil, and Korea [31, 50, 57, 62]. Isolation of VGIV is much more sporadic outside of Africa but has been associated with clinical cases in Mexico, Puerto Rico, Brazil, South America, and India [31, 56, 58, 62–64]. The sporadic cases of VGIII and VGIV appear to suggest unknown environmental reservoirs because many of these patients lacked travel history to known regions of endemic populations of VGIII or VGIV [57, 63, 65].

AFLP and PCR fingerprinting readily became the methods of choice over serotyping due to higher resolution differences observed in *C. gattii*. The major limitation of PCR-fingerprinting and AFLP analysis is that reference strains must always be included because the results are often inconsistent which can prevent direct comparisons between research groups. Technological advances and the reduced cost of DNA sequencing have led to widespread sequencing and phylogenetic analysis of *Cryptococcus.* Comparisons between strains are in some cases limited because different labs routinely utilize different primer sets. The widespread implementation of MLST analysis of *C. gattii* has expanded our understanding of its diversity [31, 66, 67]. The proposed consensus MLST scheme for *C. neoformans* and *C. gattii* put forth by the International Society for Human and Animal Mycology (ISHAM) recommends the use of 7 unlinked loci (*CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5* and the *IGS1*) for all global genotyping [68]. At each locus an allele number is assigned for each unique genetic sequence observed and sequence type (ST) is then assigned based on the combination of alleles at each locus. The allele and sequence type database for *C. gattii* is maintained and publicly accessible at http://mlst.mycologylab.org/. The MLST approach allows for the direct comparison between global isolates of *Cryptococcus* and further substantiates the recognition of the 4 major molecular types of *C. gattii* previously recognized by PCR-fingerprinting and AFLP analysis. Three additional subclades (potential varieties) within VGII/AFLP6 (VGIIa/AFLP6a, VGIIb/AFLP6b, VGIIc/AFLP6c), and VGIII (VGIIIa and VGIIIb) [26, 39, 61, 66, 69] have been observed due to MLST analysis. Global analysis of the major MLST loci has revealed no evidence for

nuclear exchange between the four VG types and these may therefore constitute four species within *C. gattii* [26, 31, 61, 66, 69]. One limitation of the currently recommended MLST scheme is that gene(s) within the mating type locus are not included, necessitating further effort to assign mating type.

The mitochondrial genomes between *Cryptococcus* species differ drastically in size and may play a role in pathogenesis [25, 26, 70]. Although gene synteny appears to be conserved, the mitochondrial genome of *C. gattii* (34.7 kb) is larger in comparison to *C. neoformans* var. *neoformans* (32 kb) and *C. neoformans* var. *grubii* (24 kb) [70–72]. A recent mitochondrial hybridization event was observed in the VGI lineage that may reflect hybridization and mitochondrial DNA exchange from VGII to VGI [70, 72]. Mitochondrial genomes of highly virulent *C. gattii* strains may be under selection because increased genetic variation was detected in coding regions versus highly conserved intergenic regions as made evident by mitochondrial recombination in *C. gattii* [70, 72].

Isolates of VGII and VGIII have been demonstrated to mate in laboratory settings but viable and fertile progeny are limited [3, 61]. Genetic rearrangements between the VG types may serve as reproductive barriers and limit productive recombination.

Molecular types, virulence, and the Pacific Northwest outbreak

MLST (multi-locus sequence typing) and VNTR (variable number of tandem repeats) analysis has distinguished *C. gattii* VGII (VGIIa and VGIIb) genotypes that are present in the Pacific Northwest [26, 61, 73]. VGIIa and VGIIb are the major and minor isolates implicated in the ongoing outbreak. The minor VGIIb genotype shows diminished virulence in comparison to VGIIa, causes a substantially smaller fraction of the outbreak-associated infections, and occurs in Australia. VGIIa and VGIIb share approximately 50% of the MLST markers analyzed, suggesting that they are related [69]. Key virulence factors like capsule, melanin, and urease are maintained between *C. gattii* molecular types, but the regulation of these factors can differ [6]. *CAS3* in *C. gattii* was not linked to capsule size *in vitro*, in contrast to *C. neoformans*, but was important for virulence of both species *in vivo* [6]. Within *C. gattii*, *CAS3* was found to be up-regulated in VGIIa in contrast to VGIIb. Microarray data identified several key virulence factors up-regulated in VGIIa compared to VGIIb that included Cas3, the Map kinase Mpk1 (involved in growth at 37°C), and the laccases Lac1 and Lac2 (involved in melanin synthesis) [6]. The original description of VGIIa in the PNW suggested this genotype may be restricted to the ongoing outbreak, but recently a VGIIa strain identical to R265 at 11 MLST loci has been reported from a cerebral cryptoccoma case in Japan. The patient reportedly traveled to Guam and Saipan and had no recent travel history to the USA [74]. The reservoir and occurrence of VGIIa outside the USA has not been determined but its isolation from an otherwise healthy Japanese patient suggests that VGIIa may be spreading, or have unknown geographic and environmental reservoirs outside of North America.

Byrnes et al. (2010) identified a new genotype, VGIIc, as a component of the outbreak expansion in Oregon. VGIIc demonstrates higher virulence similar to VGIIa in a murine model [26]. How the VGIIa genotype evolved is still unknown but two hypotheses have been advanced. The two divergent genotypes could be siblings from unknown parents, or one isolate (VGIIa) is the progeny of a mating occurring between VGIIb and another unknown isolate of the same mating type [69]. The second hypothesis for the origin of the VGIIa major genotype is that it resulted from opposite-sex mating, possibly in South America where MAT**a** strains are more prevalent. There appears to be substantial evidence for recombination occurring in natural populations that suggests the possibility of same-sex mating, opposite-sex mating, or both [26, 75]. This is further substantiated by the collection

of spore-sized particles using air samplers in the Vancouver Island region, which may be produced by mating because formation of spores requires sexual reproduction [73, 76]. The apparent absence of MAT**a** strains in the outbreak region suggests that the source of the spores, and of the recombination, is likely α-α same-sex mating. As a result of extensive environmental sampling in the PNW, one VGIIa α/α diploid strain was recovered, which supports the hypothesis that same sex mating among *C. gattii* is contributing to the outbreak [69]. Evidence of recombination occurring within the natural population also supports ongoing sexual reproduction [26, 75]. The origin of VGIIc is not yet understood. Thus far, VGIIc appears restricted and has not been isolated from patients outside Oregon or from any environmental sampling. The origin of VGIIc is unknown but could have resulted from a locally derived mutation and recombination event or from a recent introduction event. The ongoing clinical and environmental sampling and the low cost of whole genome sequencing will facilitate the sequencing of multiple strains as an alternative to MLST analysis. This approach will likely yield further understanding of the relationships between genotype, origin, and virulence of *C. gattii* [77].

Environmental distribution of *C. gattii*

Cryptococcosis is caused by the pathogenic *Cryptococcus* species including *C. neoformans* and *C. gattii*. Although attention is drawn to *Cryptococcus* as mammalian pathogens, natural populations occur as part of the environmental flora associated with trees, soil, and bird droppings. These environmental niches serve as avenues of exposure and disease acquisition because they are essentially reservoirs of the infectious yeast and spore particles. Thus, studying the distribution and pathogenicity of naturally occurring, non-clinical isolates will advance our understanding of *Cryptococcus*.

Environmental sampling involves gathering debris, decaying wood, and bird droppings, as well as swabbing various plants including, but not limited to, *Eucalyptus*, Almond, Douglas fir, Laurel, and Cacti [10, 63, 78–81]. Using conventional methods involving selective, defined nutrient media in addition to molecular analyses including MLST and AFLP markers, natural populations of *C. gattii* have been identified in both tropical and temperate regions. The global distribution of the four VG groups within *C. gattii* is summarized in Table 1. VGIV is relatively rare in non-clinical environments and most commonly found in sub-Saharan African AIDS patients, but also has been reported from Mexico, Brazil, and Puerto Rico [58, 62, 63]. VGIII is clinically prevalent in California, Mexico, was recently found as the cause of a fatal clinical case in New Mexico, and isolated from cacti in Cuba [60, 61, 82]. Although *C. gattii* is traditionally thought to be restricted to the tropics, not all tropical regions have reported natural populations. VGIII has not been isolated from indigenous trees (mopane trees) or feral pigeon droppings in South Africa [83].

Recent identification of the clinical presence of VGI in the Netherlands, Spain, Italy, and the USA in combination with successful environmental isolation from the Netherlands, Spain, Italy, India, and China is beginning to mirror the documented outbreak and expansion of the ecological niche observed for VGII in Canada and the USA [44, 46–48, 84–86]. These observations suggest an ongoing outbreak and expansion of VGI. VGI has been historically common in Australia and was found predominantly in environmental sampling across India [48, 51, 84, 85, 87]. Very little gene flow was detected between the populations of different geographic locations or between different host tree species in India, questioning the dissemination of the strains beyond their local niches. Overall, in both Australia and India, a strong clonality was found in the population structure of VGI as compared to VGII, which shows moderate signs of sexual recombination in Australia [14, 88]. VGII is very common in the PNW, Australia, and India and has recently been found in clinical and environmental isolates from Brazil [55, 89]. In Brazil, more isolates (94%) in the clinical environment were

found to be VGII than VGI (6%); but, the proportion of VGI (25%) increased in the non-clinical isolates collected in soil and bird droppings, suggesting a higher virulence of VGII in mammalian hosts [90].

Taken together, the distribution patterns of VG lineages within *C. gattii* do not fit what is expected under the "everything is everywhere" hypothesis but rather provide evidence that the VG types are endemic to specific regions of the world. Such population structure may not allow frequent gene flow, promoting speciation within *C. gattii* in nature. Nevertheless, the frequent occurrence of all VG groups except VGIII and VGIV in non-clinical environments warrants further research into the dissemination of these populations as possible sources of infection of mammalian hosts.

AIDS and *Cryptococcus gattii*

Prior to the 1950's, the incidence of cryptococcosis was relatively low and most frequently encountered in the tropics. The emergence of acquired immunodeficiency syndrome (AIDS) dramatically altered the prevalence of *Cryptococcus*. In Africa, cryptococcosis is associated with an increased occurrence of meningitis in an unusually younger cohort of patients. The increased awareness of cryptococcosis in the Congo River Basin led to the initial identification of cryptococcosis caused by an atypical strain with elongated-morphology later recognized as *C. gattii* [1, 91–93]. Today, infections caused by *Cryptococcus* are recognized as a significant cause of morbidity and mortality in HIV/AIDS patients and are annually responsible for >1,000,000 infections, >620,000 deaths, and up to one-third of all AIDS associated deaths [94, 95]. Recent estimates indicate that *Cryptococcus* is a more prevalent cause of HIV related mortality in sub-Saharan Africa than tuberculosis [95]. In other developing countries, *Cryptococcus* infections are second only to tuberculosis, and the two frequently co-occur in the AIDS population [96, 97]. The endemic, world-wide, environmental prevalence of the closely related varieties or species, *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*, has resulted in their frequent association with AIDS patients. The proportion of *Cryptococcus*/AIDS related infections that are inherently due to *C. gattii* has only recently been addressed because reporting of *Cryptococcus* infections and the determination of species is not prevalent world-wide. Retrospective and more recent clinical studies indicate *C. gattii* associated disease in AIDS patients is less common than *C. neoformans*, but the actual incidence may be dependent upon geographical origin, travel, and environmental exposure to the pathogen. *C. gattii* causes many fewer infections in AIDS patients compared to *C. neoformans*. However *C. gattii* is more prevalent than *C. neoformans* in immunocompetent and apparently healthy individuals. Serotype C, VGIII and VGIV are the most prolific *C. gattii* types associated with AIDS patients, but Serotype B, VGI, VGII and *C. gattii*-*C. neoformans* (VGI/VNI) hybrids have been sporadically isolated from AIDS patients [40, 41, 61, 98–101]. In the United States *C. gattii* molecular type VGIII (12%) is most prevalent in Californian AIDS patients. In South Africa VGIII (1%) and VGIV (1%) were found in AIDS patients [58, 61, 98, 101, 102]. In Sub-Saharan Africa, Botswana, and Malawi, VGIV was prevalent in AIDS patients (13.3%) [64]. Case reports from other parts of the world (UK, Mexico, and India) have also described *C. gattii* in HIV+ individuals [41, 103–105]. Long term outcomes of patients with AIDS and *Cryptococcus* have improved due to the decreased cost and continual development of antifungal drugs, highly active antiretroviral therapy (HAART), and combination therapy regimens, but unfortunately cryptococcal infections are still common, frequently recur, and remain difficult to cure. Key issues in undeveloped nations include difficulties in managing treatment with amphotericin B, the fact that flucytosine is not available, and that most patients receive only fluconazole, which is fungistatic and not fungicidal and can lead to drug resistance.

Sexual cycle of *C. gattii*

Cryptococcus gattii can reproduce asexually through budding and sexually through mating between the two mating types (**a**-α, opposite-sex) (Figure 1). However, natural populations of *C. gattii* predominantly contain strains of α mating type, limiting the opportunities for opposite-sex mating. This mating type is ubiquitous in all geographic regions, in both clinical and environmental isolates, and in every molecular type within *C. gattii* (VGI, VGII VGIII, and VGIV). For example, all VGI strains in India and Europe (Spain, Italy, Netherlands) and VGII strains in the PNW have the mating type α, which is also common in VGIII strains in southern California and Mexico [26, 48, 61, 87]. These observations suggest that *C. gattii*, like its sibling species *C. neoformans*, may undergo unisexual mating (α-α). In *C. neoformans*, similar environmental cues trigger opposite-sex and unisexual mating. Morphological features that distinguish the two types of mating are also known and include fused *vs.* unfused clamp connections and dikaryotic *vs.* monokaryotic hyphae (Table 2). Unisexual mating in *C. gattii* has not yet been described under laboratory conditions; however, indirect evidence has been adduced. For instance, phylogenetic incompatibilities, evidence of clonality, and limited but unambiguous evidence of recombination is found in unisexual populations of *C. gattii*, suggesting that unisexual mating may contribute to generating genotypic diversity in natural populations [106]. Here, we review the recent advancements in our understanding of the significance and frequency of opposite-sex and unisexual mating in natural populations and its impact on the pathogenicity of *C. gattii*.

Frequency of sex

The recent discovery of a sexual cycle in *C. amylolentus* indicates that virtually all species of the *Cryptococcus sensu stricto* clade can undergo sexual reproduction [107]. Studies on *C. gattii* indicate that some strains within a species could experience more sexual reproduction in nature than others. For example, strains in the VGIII lineage of *C. gattii* are highly fertile and produce more abundant spores than VGII strains. VGIII also has a higher genetic diversity, with 13 unique genotypes in southern CA, and more instances of recombination than VGII, which has a largely clonal population in the PNW. This discrepancy may reflect either a high frequency of sex in natural populations of VGIII, that VGIII was introduced to the southern California region a long time ago, or both [61]. Moreover, the VGIIIb subgroup mates more avidly under laboratory conditions and shows more signs of greater recombination than VGIIIa. The largely clonal structure of many populations of *C. gattii* may suggest either less instances of opposite-sex mating relative to asexual reproduction or frequent instances of unisexual mating between closely related or identical isolates in nature. Direct demonstration of unisexual mating in the laboratory would verify the latter possibility.

Lack of allelic exchange between the VG groups, based on MLST analyses on ~300 global isolates, is indicative of speciation [14]. VGI, VGII, and VGIII are molecularly distinct, geographically separated, which may limit opportunities for mating. Although hybridization events among *C. gattii* VG types have been demonstrated in the laboratory, limited evidence exists from nature [3, 108]. Recent MLST analysis suggests introgression events may have occurred between the VGIIIa and VGIIIb subgroups as indicated by the non-ancestral shared allele of *PLB1* [61]. *C. neoformans* hybrids with *C. gattii* VGI, VGII and VGIII also have been reported from clinical isolates, and mating has been demonstrated under laboratory conditions [40, 41, 61, 109]. It is unclear whether all VG groups are cross-fertile or mate with closely related species since fertility varies widely between isolates.

Consequences of sex

Among the pathogenic *Cryptococcus* complex, *C. gattii* is a robust pathogen of immunocompetent individuals in contrast to *C. neoformans,* which is primarily restricted to immunocompromised patients. However, less commonly infections in apparently healthy individuals by *C. neoformans* have been reported and some molecular types of *C. gattii* are more frequently associated with disease in immunocompromised individuals [61, 110–112]. The changes underlying these host shifts may include rewiring of already existing virulence machinery through sexual recombination. Hence, studying the sexual cycle in *C. gattii* may be fundamental to understanding the evolution of its pathogenicity. Sexual reproduction could contribute to the pathogenicity of *C. gattii* by generating genetically diverse progeny (basidiospores) that are also infectious propagules. In addition, spores are resistant to desiccation, are readily airborne, easily inhaled, and capable of penetrating the alveoli [113]. In Southern California, both mating types are present in VGIII clinical populations, suggesting the possibility for opposite-sex mating in nature [14]. Several of these isolates were indeed found to be highly fertile in laboratory tests, producing abundant spores. In populations where only one mating type predominates, unisexual mating may generate similar infectious propagules in *C. gattii*.

The process of meiotic recombination during mating may bring together useful gene combinations, producing progeny better suited to expand the host range including immunocompetent individuals and temperate regions as implicated in VGII isolates found in the PNW. Clinical isolates of the VGII lineage also show higher minimum inhibitory concentrations for azole drugs and may require more aggressive and longer term treatment [14, 114]. Sex may also help generate greater variance in virulence characteristics. For example, in the VGIII lineage, which is generally very fertile, frequent recombination may have created larger variation in virulence with some progeny emerging as more virulent and others less virulent. Similarly, the subgroup VGIIIb is more fertile and less virulent as compared to VGIIIa, both in murine models and IPR assays [61]. The VGII and VGIIIa genotypes may represent highly virulent sexual progeny whose virulence could be maintained through clonal reproduction. Both VGII and VGIIIa lineages contain isolates almost exclusively of α mating type. Consequently, unisexual mating may also contribute to maintaining the gene combinations responsible for increased virulence.

Lastly, a direct link between mating and virulence pathway is possible because the mating type locus (*MAT*) may directly contribute to regulation of virulence. The *MAT* locus regulates the response to mating pheromones and subsequent hyphae and basidiospore formation. Previous studies in *C. neoformans* have shown that congenic strains, which differ only at the *MAT* locus, differ in virulence, indicating that *MAT* contributes to pathogenicity of the species [109, 115–117]. Additional investigations observed no difference in virulence between strains of opposite mating types indicating that the influence of *MAT* depends on the genetic background [117–119]. Virulence is a polygenic trait and the *MAT* locus governs virulence in concert with other genes, stressing the importance of genetic background in regulation of virulence. The *MAT* locus in the VGIII lineage exhibits genomic plasticity including gene rearrangements, truncation, and gene loss [61]. The observed plasticity may be a direct cause or a consequence of the associated high fertility of VGIII. *C. gattii* has a bipolar *MAT* locus similar to its pathogenic sister species, *C. neoformans*. Studies on closely related non-pathogenic species of *Cryptococcus* suggest that the transition to bipolarity occurred recently and concomitantly with the emergence of the pathogenic *C. neoformans/C. gattii* species cluster [107]. Studying the structure and function of the *MAT* loci of various clinical and non-clinical isolates and its association with virulence is necessary to establish the role of mating in the regulation of pathogenicity in *C. gattii*.

Conclusion

Research has demonstrated that *C. gattii* is more than just an opportunistic pathogen because it harbors distinct mechanisms geared to evade, subvert, and manipulate the host immune system, while maintaining intracellular growth. *Cryptococcus gattii* is likely to have a wider geographical distribution than presently appreciated based on the ongoing expansion of clinical and environmental reports worldwide. Historically, the prevalence of *C. gattii* has been overlooked because most clinical laboratories did not distinguish *C. gattii* from *C. neoformans*. Due to the increasing documentation of *C. gattii* in previously undocumented regions, observed differences in drug sensitivity, and disease outcome, clinicians should pursue species-specific diagnoses for all cases presenting with cryptococcosis. CGB agar and MLST analyses are quick, inexpensive, and reliable methods of distinguishing *C. gattii* from *C. neoformans* and should be incorporated into standard clinical testing procedures for cryptococcosis.

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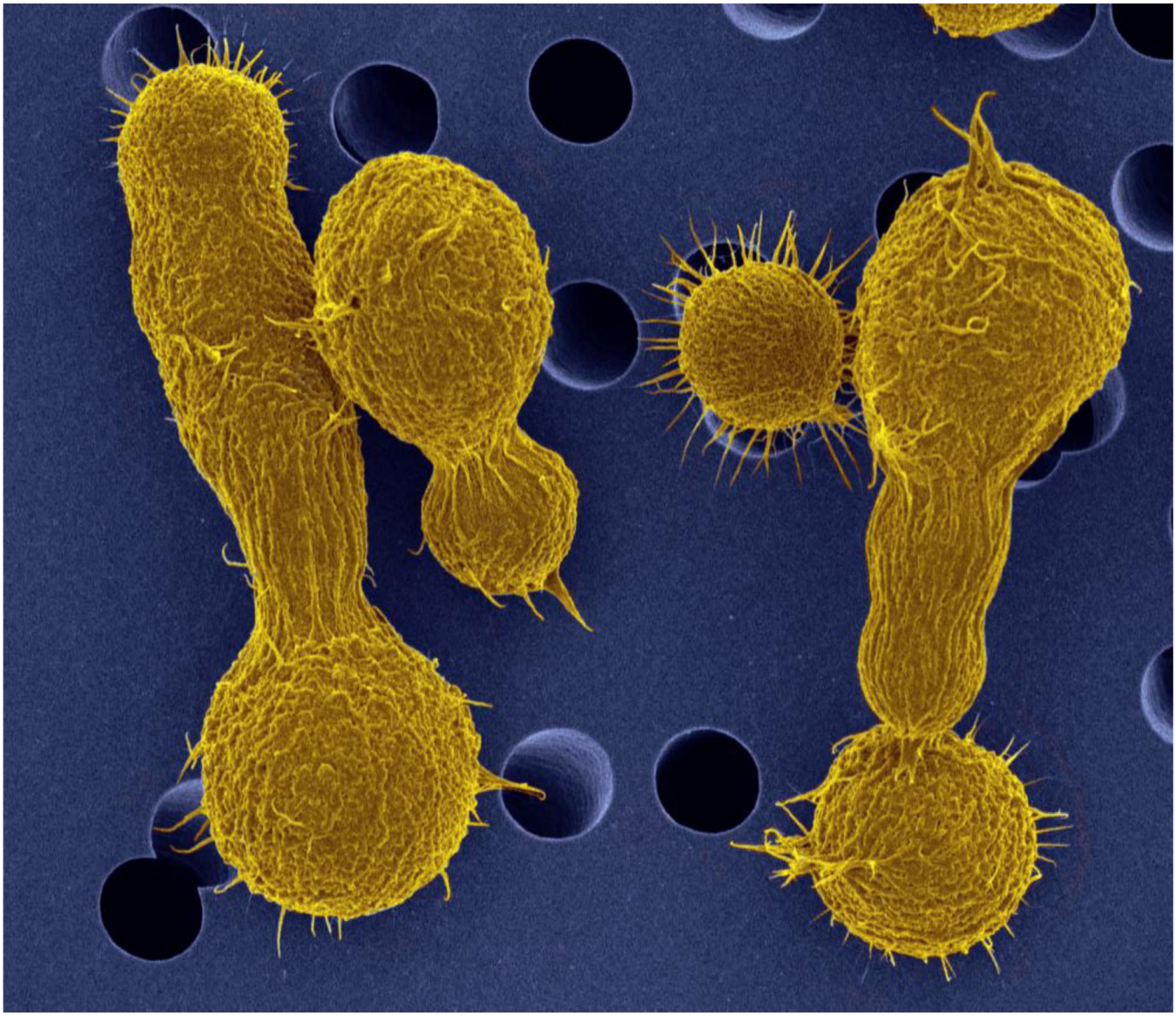

Figure 1.
Pseudo-colored image of *Cryptococcus gattii* yeast cells demonstrating elongated-cell morphology. “Original black and white SEM image Copyright Dennis Kunkel Microscopy, Inc , pseudo colored image by Deb Springer.”

Table 1

Cryptococcus molecular types, geographic and clinical distribution

Species/Hybrids*	Geographic distribution	Group	Source	Patient risk	Reference Strains
C. neoformans var. *grubii*	Most common worldwide	VNI	Clinical, Veterinary, & Environmental	world-wide in compromised, immunocompetent China and Korea	H99(α); 125.91 (MAT**a**)
	Aftrica	VNB	Clinical	compromised, rare in immunocompetent	BT148(α); BT63 (MAT**a**)
	Australia	VNII	Clinical, Veterinary, & Environmental	compromised, rare in immunocompetent	8-1(α)
C. n. var. *grubii* -*C. n.* var. *neoformans**	Europe, South America	VNIII	Clinical	compromised, rare in immunocompetent	KW5αADa; CDC228
C. neoformans var. *neoformans*	Europe, South America	VNIV	Clinical, Veterinary, & Environmental	compromised, rare in immunocompetent	JEC21(α); JEC20 (MAT**a**)
C. gattii	Most comon *C. gattii* , Europe, less common in Africa, South America, North America, Australia	VGI	Clinical, Veterinary, & Environmental	Immunocompetent, rare in compromised	WM276(α); E566 (MAT**a**)
	North and South America, Australia, less common in Oceania	VGII	Clinical	Imunocompetent	WM178(α); CBS1930 (MAT**a**)
	North America, reported from Asia, Australia, Europe, South America	VGIIa	Clinical, Veterinary, & Environmental		R265(α)
	Northwestern USA, Australia, Southwestern Canada	VGIIb	Clinical, Veterinary, & Environmental		R272(α)
	Oregon, USA	VGIIc	Clinical & Veterinary		EJB18(α)
	South America less common in Noth America, Australia, Europe, Africa	VGIIIa	Clinical, Veterinary, & Environmental	Immunocompetent, HIV+	WM161(α); CA1872 (MAT**a**)
		VGIIIb	Clinical		NIH312(α); B4546 (MAT**a**)
	Africa, India, South America	VGIV	Clinical, Veterinary, & Environmental	Immunocompetent, HIV+	BT26; WM779(α)
C. n. var. *neoformans(VNIII)* -*C. gattii* (VGI)*	Africa, Netherlands, South America	NA	Clinical	Compromised	CBS10488, CBS10489, CBS10490
C. n. var. *grubii (VNI)*-*C. gattii* (VGI)*	Europe, North America				CBS10496

Table 2

Features distinguishing unisexual and opposite-sex reproduction in *C. neoformans* (Lin et al 2005)

Feature	Unisexual reproduction	Opposite-sex reproduction
Mating types involved	α	**a** and α
Hyphae	Monokaryotic	Dikaryotic
Clamp connections	Unfused	Fused
Blastospores	Haploid or diploid, always α	Haploid or diploid, could be **a** or α
Spores	All α	**a** and α in a 1:1 ratio