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Article

Phylogenomic Placement of American Southwest-Associated Clinical and Veterinary Isolates Expands Evidence for Distinct *Cryptococcus Gattii* VGVI.

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Abstract: Whole-genome sequencing has advanced our understanding of the population structure of the pathogenic species complex *Cryptococcus gattii*, which has allowed for the phylogenomic specification of previously described major molecular type groupings and novel lineages. Recently, isolates collected in Mexico in the 1960s were determined to be genetically distant from other known molecular types and were classified as VGVI. We sequenced four clinical isolates and one veterinary isolate collected in the southwestern U.S. and Argentina during 2012-2021. Phylogenomic analysis groups these genomes with those of the Mexican VGVI isolates, expanding VGVI into a clade and establishing this molecular type as a clinically important population. These findings also potentially expand the known *Cryptococcus* ecological range with a previously unrecognized endemic area.

Keywords: *Cryptococcus*; Whole-Genome Sequencing; VGVI; phylogenomics; Molecular Type

1. Introduction

Whole-genome sequencing has provided an important element in defining the population structure for many human fungal pathogens including the *Cryptococcus* species complexes (*C. gattii* and *C. neoformans*). However, genomic tracking of *Cryptococcus* is seldom done for the estimated 223,100 annual clinical meningitis cases worldwide [1]. This dearth is even more pronounced for veterinary cases. Epidemiological and academic research efforts using methods such as restriction fragment length polymorphism (RFLP), multilocus sequence typing (MLST), and most recently, whole-genome sequencing [2] have defined the *Cryptococcus* subpopulations into molecular types, also proposed to be separate species [3]. The most prevalent *C. gattii* populations causing illness have been characterized and sequenced, and comprise mostly the molecular types VGI, and VGII, although the VGIII [4] and VGIV[5] genomic populations have also been well described. Because of increased collection and genome sequencing of isolates around the world, emerging and less prevalent popu-

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lations have recently been added to the species phylogenomic tree. Such is the case for *C. gattii* molecular type VGV, found through environmental sampling in central Zambian woodlands [6], as well as the new lineage VGVI represented by multiple laboratory strains derived from one or two clinical cases in Mexico from early 1960's [6] and an isolate from a Mexican immigrant living in Spain from 1987 [8]. These clonal VGVI isolates were previously proposed to be a distinct species, named *C. decagattii* [3].

We sequenced four clinical isolates and one veterinary isolate of *C. gattii* collected during 2012-2021 in the Southwest U.S. and Argentina. Here we present the genomes of these isolates and their phylogenomic placement in the species tree.

2. Materials and Methods

Of the five isolates of *C. gattii* sequenced in this study, three were collected from patients with pulmonary or meningeal cryptococcosis in Arizona and classified as *C. gattii* by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Table 1). The geography and coincidence of the clinical cases prompted further investigation. The remaining two isolates were selected for sequencing from the Westmead Medical Mycology Collection, Sydney/Perth, Australia due to their MLST profiles. These were collected from a clinical case in Argentina [7] and a veterinary case in Arizona (Table 1).

Table 1. Case information for *C. gattii* VGVI genomes. Definitions: CHF, congestive heart failure; CSF, cerebrospinal fluid; ND, not determined

ID	Isolate source	Collection locale	Collection Year	Sample type	Comorbidity	Cryptococcosis presentation	WGS source	Metadata source
AZ04665	Clinical	USA/AZ	2019	CSF	HIV/AIDS	Meningitis	This Study	This study
AZ92981	Clinical	USA/AZ	2019	Blood	Alcoholic cirrhosis and hepatitis, CHF	Pulmonary	This study	This study
AZ00135650	Clinical	USA/AZ	2021	CSF	Pulmonary lesions/ Hx of Lymphoma	Bronchitis/ Meningitis	This study	This study
WM 11.135	Veterinary	USA/AZ	2011	Nasal	Possible underlying hepatopathy	Upper respiratory signs	This study	This study
WM 20.07	Clinical	Argentina/ Salta	2017	CSF	Malnutrition	Meningoencephalitis	This study	[7]
WM 18.02	Clinical	Mexico/DF	1961	CSF	ND	Meningitis	[4]	[8]
WM 18.04	Clinical	Mexico/DF	1965	CSF	ND	Meningitis	[4]	[8]
CBS 11687	Clinical	Mexico*	1987	ND	ND	ND	[6]	[9]

*The isolate was obtained from a Mexican non-HIV patient living in Spain

Genome libraries were prepared with a DNA prep kit (Illumina) at 1/4th of the reaction volumes and sequenced on a NextSeq 550 (Illumina). Molecular typing was performed over the sequence reads via a custom 31-mer Kraken2 database [10] consisting of publicly available genomes from representatives of each molecular type of the *C. gattii* species complex, including the "VGVI" genomes originating from Mexico in the 1960s and 1987

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(SRA: SRS1519517, SRR3707827, SRR7345539). Read files for each sample would be considered typed when read-counts assigned to the top match in the custom database surpassed those of the second-best match by a factor greater than two. Draft genomes were assembled to be used as references for single nucleotide polymorphism (SNP) detection using SPAdes v3.10.1 with the "careful" flag activated [11].

Subsequently, sequence reads were compared to public genomes, including that of their putative molecular type VGVI (AZ04665 draft assembly used as alignment reference) by SNP-based phylogenetic inference. Reads were mapped to reference genomes using BWA v0.7.17 [12] and SNPs called using GATK unifiedGenotyper v3.7 [13] using the bioinformatic pipeline NASP [14]. The SNP matrix output was filtered to include only positions where all genomes had ≥10X depth of reads and a read agreement proportion of ≥0.9 to produce high-confidence SNPs. The concatenated SNP profiles of each genome were used to perform maximum parsimony inference using Phangorn [15] to generate phylogenomic trees using the ratchet method [16] from a 75% consensus of 30 ratchet trees produced from initial topologies found through random generation or random addition. Five hundred Bootstrap replicates generated the confidence values for each node. Final phylogenies were plotted using ggtree [17]. Sequence data from this project are available in NCBI Bioproject PRJNA826887.

3. Results

Genomes for the five isolates matched the VGVI reference genomes through k-mer content classification. Phylogenomic reconstruction confirmed that they were more related to the originally named VGVI isolates [6] than other *C. gattii* molecular types (Figure 1A). Mean pairwise distances within the VGVI clade are ~11.5 times shorter than the mean pairwise distance to the tips of the next major molecular type (VGIII), establishing VGVI as reciprocally monophyletic and distinct from other populations. The phylogenomic analysis of the VGVI genomes reveals approximately one hundred thousand high-confidence SNPs among them (Figure 1B); the long branches indicate a population structure with a deep (i.e., not recent) evolutionary history. The Argentinian genome WM 20.07 is basal to the rest of the recently collected samples yet more related to these than to the older WM 1804, WM 1802, and CBS 11687 collected from Mexican patients.

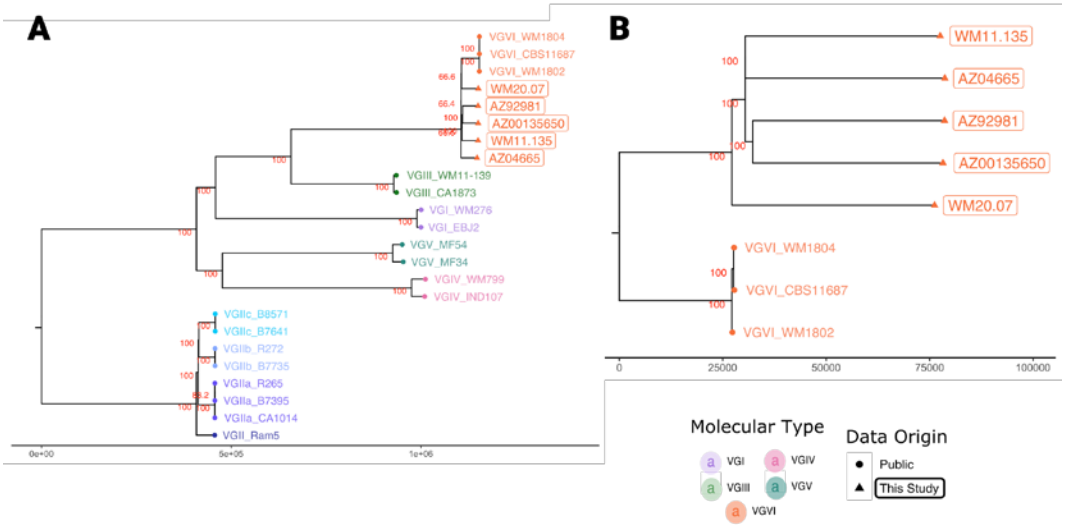

Figure 1. Maximum parsimony phylogenies based on high confidence SNPs showing boxes around the isolates sequenced in this study. A) This analysis of 24 genomes includes two to eight publicly available genomes from each of the major *C. gattii* molecular types. The tree covers 87.40% of the

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reference and comprises 1,733,389 polymorphic loci, with a consistency index of 0.88 and a retention index of 0.97. B) The VGVI-only phylogeny includes all 8 available genomes for this molecular type and comprises 1,123,371 polymorphic loci covering 91.88% of the reference genome. This phylogeny has a consistency index of 0.78 and a retention index of 0.73. The reference for both trees was AZ04665 draft assembly (17.5 Mbp N50: 185.1 Kbp).

4. Discussion

In recent years, outbreaks of fungal disease affecting humans and animals have manifested previously uncharacterized taxa and challenged our knowledge of fungal disease epidemiology and biogeography. Such was the case for the global emergence of *Candida auris* [18] and for the appearance of *C. gattii* in the Pacific Northwest [4,19]. The five isolates sequenced in this study, four of which originate from human/veterinary clinical cases in warm, arid regions of the U.S. desert southwest, similarly expand *C. gattii* taxonomy and potentially its biogeography, by establishing VGVI as a distinct population of *C. gattii* with a long evolutionary history.

The origins of the isolates comprising VGVI suggest a new endemic area that may include the southwestern U.S. and Mexico, as well as regions of Argentina. If indeed this population inhabits the American Southwest, several questions about adaptations of VGVI to warm, arid environments are raised; this includes whether VGVI has departed from traditional niches for *C. gattii*, usually found in moist and nutrient-rich microenvironments. Dry soils in temperature-extreme environments are generally unexpected places to find fungi [20]; however, microenvironments like the extensive cryptobiotic grounds in the southwest U.S. [21,22] and organism adaptations like those of *Coccidioides* spp.—both at the cellular [23,24] and life cycle levels (e.g. soil sterilization hypothesis [25,26] and small-mammal reservoir hypothesis [27,28])—illustrate the clear possibility of the presence of other cryptic environmental fungi in these ecosystems. Additionally, other desert-adapted yeasts also exemplify survival strategies that may be suited to *C. gattii*. Such is the case for the cactophylic *Sporopachyderma* spp. which have been collected in southern Arizona dwelling in saguaro cactus soft rot pockets [29,30] and have been reported to be rare opportunistic human pathogens[31,32]. Of note, all known VGVI isolates were collected exclusively from clinical or veterinary infections. Environmental isolation will be an important step in elucidating the niche of VGVI and its endemicity in the region.

The basal placement of the Argentinian isolate in the VGVI phylogeny shows the immediate relation of the isolate to the North American cases and indicates that *C. gattii* VGVI is a possible risk for patients in previously undescribed endemic regions of the globe. A lack of clear exposure history for the Arizona human patients prevents confirmation of endemic locales at this time. Nevertheless, the link to the southwestern US is compelling when considering the provenance of the isolates; for example, clinical isolate 7685027 collected in southern California has been reported by Springer *et al*. [33] which likely also belongs to this clade since its MLST profile is very similar to that of CBS 11687 [3]. The locale of the veterinary cases is of extreme interest due to limited travel distances for pets. An additional veterinary case of *C. gattii* in Arizona has been previously published [8] which may lead to additional epidemiological follow-up. Further genomic analysis of potential VGVI isolates, especially veterinary and environmental isolates, may clarify the biogeography, epidemiology, and potential health risk of this molecular type of *C. gattii*.

The VGVI population structure shows substantial differences across samples (Figure 1b), which contrasts with the close relatedness of the original three VGVI genomes. According to metadata published in 2003 [8], the samples WM 1802 (LA 390, INDRE 5604) and WM 1804 (LA 392, INDRE 5606)) were collected in 1961 and 1965 respectively from disparately aged patients (38 and 40), whereas CBS 11687 (IHEM 14941S) published records indicate collection in 1987 in Europe from a Mexican immigrant [3,9]. This contradicts

a previous hypothesis proposing these were derived from a single isolate [6]. Additional high-resolution SNP analysis of only these three genomes (data not shown) resulted 144 and 149 high-confidence SNPs between CBS 11687 and the other two isolates, which in turn are differentiated by 21 bases between them. These relatively few differences explain the reduced diversity measurements for VGVI and provide further evidence that isolates either represent a single originating clone with subsequent laboratory acquired mutations, or they independently originated from the same exposure source. In contrast to the reduced genomic diversity of the isolates that first defined VGVI, the three clinical isolates recently collected in Arizona in 2019 and 2021 are divergent with ~100,000 high-confidence SNPs between samples. This reinforces the case for a deep evolutionary history that we have yet to uncover and underlines the likelihood of past clinical cases that went mischaracterized.

Cryptococcus molecular typing is rare in clinical settings, but new technologies make it a possibility now at hand. The use of MALDI-TOF MS in the Arizona clinical microbiology laboratory in this study is a recent change. Previous methods may have misclassified *C. gattii* infections as *C. neoformans*, the more likely species to be encountered in this setting. The use of MALDI-TOF MS for rapid, simple, and reliable molecular type identification in *Cryptococcus* spp. has been demonstrated in research applications [3,34,35] and could potentially be used to rapidly screen for suspect VGVI isolates. Better isolate typing is an important development that can spark further research providing concurrent and retrospective isolates to fill the gaps in our understanding of molecular type VGVI.

Tracking the geographical origin and the dynamics of spread is especially challenging in fungi due to their complex ecology and the large timescales, usually, millennia, that encompass the evolution of these organisms [4,36]. Reconstructing likely scenarios for spread is essential to understanding disease emergence and risks to public health; both natural history and human commercial activities have been correlated with the emergence of fungal etiologic agents and fungal resistance, such as in *Candida auris* [37,38] and *C. gattii* VGII [39]. These advances/theories are particularly reliant on whole-genome sequencing and molecular clock analyses that reveal the time scale and patterns of evolution; these efforts are ongoing for *C. gattii* VGVI. Further surveillance that includes typing methods, especially whole-genome sequencing, will provide the opportunity to clarify the endemicity of VGVI and whether this pathogen represents an emerging risk to susceptible human and animal hosts in the identified regions.

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Data Availability Statement: All sequence data generated and used is made public through NCBI. Sequence reads produced for this study can be found in Bioproject PRJNA826887.

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