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# Identification of Novel Hybrids Between *Cryptococcus neoformans* var. *grubii* VNI and *Cryptococcus gattii* VGII

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**Abstract** *Cryptococcus neoformans* and *Cryptococcus gattii* are pathogenic yeasts causing meningoencephalitis in immunocompromised and immunocompetent hosts. The fungus is typically haploid, and sexual reproduction occurs normally between individuals with opposite mating types, α and **a**. *C. neoformans* var. *grubii* (serotype A) is comprised of molecular types VNI, VNII, and VNB, and *C. neoformans* var. *neoformans* (serotype D) contains the molecular type VNIV. Additionally, diploid or aneuploid AD hybrids (VNIII) have been reported. *C. gattii* contains the molecular types VGI, VGII, VGIII, and VGIV, which encompass both serotypes B and C. To identify possible hybrid strains, *URA*5-RFLP analysis was performed on 350 globally obtained clinical, environmental, and veterinary isolates. Four clinical isolates from cerebrospinal fluid showed combination patterns of *C. neoformans* var. *grubii* and *C. gattii*: Brazil ($n = 2$), Colombia ($n = 1$), and India ($n = 1$). These strains were monokaryotic and diploid or aneuploid. M13 PCR fingerprinting showed that they contained fragments of both proposed parental groups. Luminex IGS genotyping identified these isolates as hybrids with two different molecular type combinations: three VNI/VGII and one VNI/VGI. Blue color development on CGB agar was delayed in three isolates and absent in one. *C. gattii*-specific PCR confirmed the presence of *C. gattii* in the hybrids. *CAP59* allele-specific PCR revealed that all the hybrids contained both serotype A and B alleles. Determination of mating-type allelic

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patterns by PCR revealed that the isolates were αA **a**B. This is the first study discovering novel natural hybrids between *C. neoformans* molecular type VNI and *C. gattii* molecular type VGII.

**Keywords** *Cryptococcus neoformans* · *Cryptococcus gattii* · AB hybrids · Serotype · Mating type · Molecular type

## Introduction

*Cryptococcus neoformans* and *Cryptococcus gattii* are encapsulated basidiomycetous yeasts that cause meningoencephalitis. *C. neoformans* is an opportunistic pathogen, causing disease in immunocompromised patients [1]. *C. gattii* is considered as a primary pathogen, as it typically affects immunocompetent hosts [2]. *C. neoformans* has a worldwide distribution, and its major natural reservoir is pigeon droppings [3], soil contaminated with avian guano [4], and decaying wood of many tree species [5]. *C. gattii* is associated with decaying wood, plant debris, and soil [6, 7]; its distribution is limited, but includes tropical or subtropical areas [8] and certain temperate zones in Australia [9], Canada [7, 10], Greece [11], Italy [12], South Africa [13], Spain [14], and the North-West of the USA [15].

*Cryptococcus* is typically a haploid fungus with a bipolar heterothallic mating system containing one mating-type locus, which occurs in either of the two alleles, **a** or α. Sexual reproduction normally occurs between opposite mating types [16]. *C. neoformans* is comprised of two varieties: *C. neoformans* var. *grubii* (serotype A, molecular types VNI & VNII) and *C. neoforman* var. *neoformans* (serotype D, molecular type VNIV). In addition, diploid and aneuploid AD hybrids have been reported in clinical and environmental samples [1, 17]. Recently, based on a multilocus sequence typing (MLST) method, a new molecular type (VNB) has been identified among African and South American isolates of serotype A [18, 19]. *C. gattii* is comprised of serotypes B and C, and contains molecular types VGI, VGII, VGIII, and VGIV [17, 20]. Overall, serotype A isolates are the most common pathogens, being responsible for over 90% of cases of HIV-associated cryptococcosis worldwide [21–23].

In addition to AD hybrids, several inter- and intra-varietal diploid or aneuploid hybrids have been reported in the *C. neoformans*/*C. gattii* species complex [24–27]. Recently, three BD *C. neoformans* var. *neoformans* VNIV × *C. gattii* VGI hybrids from two HIV-negative patients in the Netherlands and one AB *C. neoformans* var. *grubii* VNI × *C. gattii* VGI hybrid from an HIV-positive patient in Quebec, Canada, have been described [24, 25]. Intra-varietal AA hybrids between VNII and VNB have also been reported [26, 27].

In the present study, a random selection of global clinical, environmental, and veterinary isolates maintained in the culture collection of the Molecular Mycology Research Laboratory at Westmead Hospital, Australia, was investigated to detect the presence of possible hybrids between the two species *C. neoformans* and *C. gattii* and characterize them in order to better understand the overall population structure of this important human/animal pathogen.

## Materials and Methods

### Clinical and Reference Strains

A random selection of 350 global *C. neoformans* and *C. gattii* isolates of clinical, environmental, and veterinary origins was obtained from the culture collection of Molecular Mycology Research Laboratory at Westmead Hospital, Australia, and screened for potential hybrid strains. Eight laboratory standard reference strains representing each molecular type, WM 148 (VNI, serotype A), WM 626 (VNII, serotype A), WM 628 (VNIII, serotype AD), WM 629 (VNIV, serotype D), WM 179 (VGI, serotype B), WM 178 (VGII), WM 175 (VGIII), and WM 779 (VGIV, serotype C) [17], were used for the identification of the major molecular types. The strains H99 (*MAT*α, serotype A) [28], IUM 96-2828 (*MAT***a**, serotype A) [29], JEC21 (*MAT*α, serotype D) [30], JEC20 (*MAT***a**, serotype) D [32], and WM 04.79 = LA599 (VGII *MAT***a**) [31] were used as controls for mating, serotype identification, luminex, and flow cytometry experiments.

### Genomic DNA Extraction

Isolates were grown on Sabouraud's dextrose agar at 30°C for 48–72 h. Genomic DNA was then extracted according to Ferrer et al. [32]. Prior to performing

PCRs, the DNA was diluted in sterile deionized water to a concentration of 10 ng/μl.

*URA5*-RFLP Analysis

The *URA*5 gene of all 350 isolates was amplified with primers URA5, 5′-ATGTCCTCCCAAGCCCTCG ACTCCG-3′ and SJ101, 5′-TTAAGACCTCTGAA CACCGTACTC-3′, as previously described by Meyer et al. [17]. PCR products were digested with *Hha*I and *Sau*96I enzymes at 37°C over night. Digestion products were separated by 3% agarose gel electrophoresis and molecular types were identified by comparison of each profile with those of the eight standard reference strains [17]. Isolates with mixed patterns were considered to be potential hybrids.

Ploidy Determination by Fluorescence Flow Cytometry

Flow cytometry was performed on 10,000 cells of each potential hybrid isolate as described previously [33] and analyzed with a Becton–Dickinson FACSCallibur (BD Biosciences, San Jose, CA, USA). Cells stained with propidium iodide were examined by fluorescent microscopy to check the number of nuclei per cell.

Luminex Suspension Array

Oligonucleotide probes targeting either *C. neoformans* or *C. gattii*, specific oligonucleotide probes for serotype A and D, plus four probes for each of the molecular types of *C. gattii* were used as described by Diaz and Fell [34]. In addition, to separate VNI and VNII molecular type, two new specific probes for each VNI and VNII molecular types were designed based on sequence data from the IGS1 region of the ribosomal DNA, CNN 1a/1b: 5′-GGAATAAGGGA TGGATAAA-3′ and CNN 1c: 5′-GGAATAAGG AATGGATAAA-3′. Luminex analysis was performed on potential hybrid isolates as described by Diaz and Fell [34], Diaz et al. [35], Fulton et al. [36] and Diaz and Fell [37]. Detection analysis was carried out on the Luminex 100 analyzer (Luminex Corporation, Austin, TX, USA), a flow cytometer with a dual laser system that allows simultaneous bead set recognition and quantification of targets based on the fluorescence of the reporter molecule. One hundred microspheres of each set were analyzed, which represents 100 replicate measurements. Median fluorescent intensity (MFI) values were calculated with a digital signal processor and the Luminex proprietary software. A blank (without sample DNA) and a negative control (unrelated DNA) were included in the assay to ensure that sample contamination during the reaction will not result in a false-positive interpretation of the data. A positive signal was defined by a signal intensity at least twice the background or blank MFI value after subtraction of the background. The experiment was run twice.

M13 Fingerprinting

The minisatellite-specific core sequence of the wild-type phage M13 (5′-GAGGGTGGCGGTTCT-3′) [38] was used as a single primer in the PCR, previously described by Meyer et al. [17]. Amplification products were separated by electrophoresis on 1.4% agarose gels, containing 0.5 μg/ml ethidium bromide in 1× Tris–borate-EDTA (TBE) buffer (89.1 mM Tris base, 88.9 mM Boric acid, 50 mM EDTA, pH 8.0) at 70 V and visualized under UV light. The 1-Kb DNA Ladder (Invitrogen, Carlsbad, USA) was used as a molecular size marker. WM 148 (VNI), WM 626 (VNII), WM 179 (VGI), and WM 178 (VGII) were used as reference strains.

Growth on CGB Medium

Hybrid isolates were grown at 27°C on canavanine-glycine-bromothymol blue agar (CGB) medium, which distinguishes between *C. gattii* (cobalt blue) and *C. neoformans* (yellow) growth, due to a pH-dependent color change [39]. The color of CGB medium was assessed after 5 and 15 days incubation, since it was predicted that the blue color development would be delayed in hybrid strains. Strains WM 148 (VNI) and WM 179 (VGI) were used as controls.

*C. gattii*-Specific PCR

To confirm the presence of *C. gattii* in the putative hybrids, *C. gattii*-specific PCR was performed using the 660 U and 660 L *C. gattii*-specific primers, which are based on an anonymous DNA fragment (216 bp) that was amplified by randomly amplified polymorphic DNA (RAPD) analysis from a *C. gattii* strain [40]. 10 μl of each amplification product was electrophoresed at

80 V on 2% agarose gel in 1× TBE buffer containing 0.5 μg/ml ethidium bromide and visualized under UV light. The 1-Kb Plus DNA Ladder (Invitrogen, Carlsbad, USA) was used as a molecular size marker. WM 148 (VNI) and WM 179 (VGI) were used as control strains.

*CAP59* Allele-Specific PCR

To identify the serotype-specific allele combination in the potential hybrids, the *CAP59* gene was amplified with primers CH-Cap 59F and CH-Cap 59R, as described previously [41]. Amplicons were subjected to analysis using three separate restriction enzymes—*Bsm*FI, *Hpa*II, and *Age*I—according to the instructions of the manufacturer (New England Biolabs, Ipswich, MA, USA). Digested fragments were separated and visualized on a 3% agarose gel stained with ethidium bromide. Enzymatic restriction with *Bsm*FI and *Hpa*II distinguishes between the A, D, and B/C alleles. Serotype AD strains exhibit a mixture of A and D restriction profiles. The B and C alleles can be differentiated by *Age*I digestion [41].

*GPA1* and *PAK1* Allele-Specific PCR

The presence or absence of serotype A- and D-specific alleles of the *GPA1* gene was determined by PCR using the primer sets JOHE2596/JOHE3241 specific for serotype A allele and JOHE2596/JOHE3240 specific for serotype D allele [42], and for *PAK1* gene it was determined using the primer sets JOHE3066/JOHE3236 specific for serotype A allele and JOHE3066/JOHE3065 specific for serotype D allele [43], as described previously. The following cryptococcal strains were used as control strains for different serotypes: H99 (serotype A), JEC21 (serotype D), WM 179 (serotype B), and WM 779 (serotype C).

Determination of Mating-Type Allelic Partners

To determine the mating-type allelic partners of the potential hybrid strains, the *MFα/a* locus was amplified with the primer sets MFαF/MFαR and MFaF/MFaR [44], the *STE12α/**a*** locus was amplified with the primer sets MFαU/MFαL [40] and JOHE9779/JOHE9780 [10], the *STE20**a*** locus was amplified with the primer set JOHE9421/JOHE9422 [10], the Aα allele of the *STE20α/**a*** locus was amplified with the primer set JOHE7264/JOHE7266 [43], the Dα allele of the *STE20α/**a*** locus was amplified with the primer set JOHE7267/JOHE7269 [43], the A**a** allele of the *STE20α/**a*** locus was amplified with the primer set JOHE7270/JOHE7271 [43], the D**a** allele of the *STE20α/**a*** locus was amplified with the primer set JOHE7273/JOHE7274 [43], the Aα allele of the *SXI1α/2**a*** locus was amplified with the primer set JOHE15634/JOHE15635 [43], and the Dα allele of the *SXI1α/2**a*** locus was amplified with the primer set JOHE15634/JOHE15636 [43], as previously published. The following cryptococcal strains were used as control strains for the two mating types: H99 (Aα), IUM 96-2828 (A**a**), JEC21 (Dα), JEC20 (D**a**), WM 179 (Bα), and WM 04.79 (VGII *MAT***a**).

## Results

Mixed patterns of both *C. neoformans* var. *grubii* and *C. gattii* were observed in cerebrospinal fluid isolates from four patients with cryptococcal meningitis. Comparison of the *URA5*-RFLP profiles of all 350 isolates with those obtained from the standard reference strains revealed mixed patterns of VNI/VGI for one isolate (WM 2617) from a patient in India and VNI/VGII for three isolates (WM 05.272, WM 05.459, and WM 05.532) from patients in Colombia and Brazil (Fig. 1a; Table 1).

Determination of ploidy using flow cytometry analysis showed that all potential hybrid isolates contained ∼2× the amount of DNA in the haploid control strain, confirming that they were diploids or aneuploids (Fig. 2). All cells were uninucleate.

The putative hybrids were analyzed using Luminex xMAP technology, a flow cytometer that allows the simultaneous identification of cryptococcal varieties and their genotypes by mixing different sets of microspheres containing specific capture probes derived from IGS target sequences. The *C. neoformans* probe (CNN b), *C. neoformans* var. *grubii* probe (CNN 1b), *C. gattii*-specific probe (CNG), and the *C. neoformans* var. *grubii* VNI probe (CNN 1a/1b) gave a positive signal for all four *C. neoformans/C. gattii* hybrid isolates. Additionally, for WM 05.272, WM 05.459 and WM 05.532 positive signals were observed from VGII-specific probe (CNG 3), and for WM 2617, the probe specific for VGI (CNG 4c) gave positive signals (data not shown). Therefore, this technique

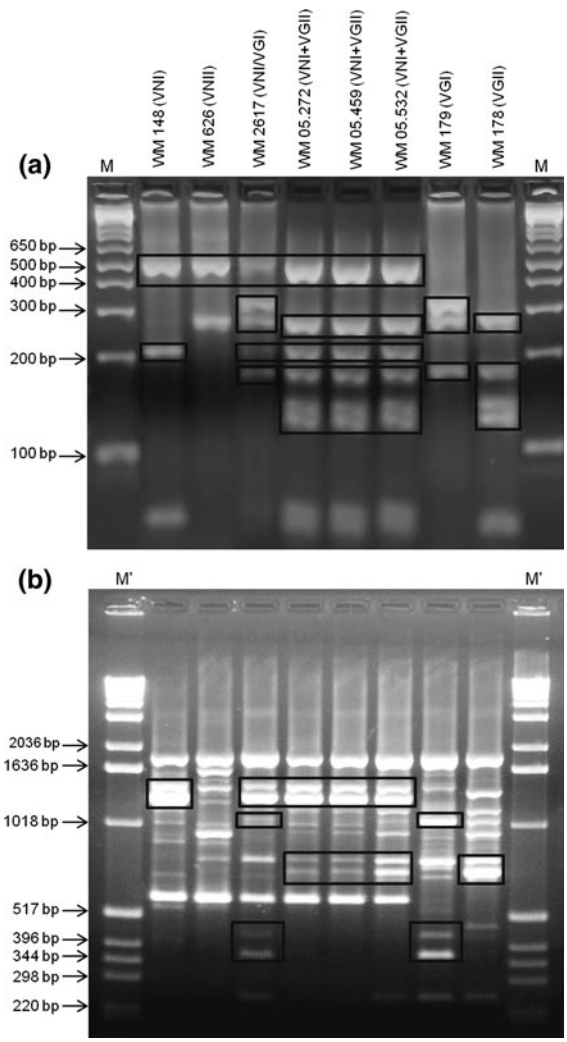


**Fig. 1** **a** RFLP profiles of the putative hybrid isolates as well as standard strains for all molecular types obtained via double digest of the *URA*5 gene with *Hha*I and *Sau*96I. *Boxes* indicate fragments occurring both in hybrid isolates and their parental groups. *Lane M*, 1-kb$^{+}$ DNA ladder (Invitrogen, Carlsbad, USA). **b** M13 PCR fingerprint of the putative hybrid isolates and the standard strains for each of the putative parental groups. *Boxes* indicate fragments occurring both in hybrid isolates and their parental groups. *Lane M′*, 1-kb DNA ladder (Invitrogen, Carlsbad, USA)

identified the isolates as hybrids with two different molecular type combinations, VNI/VGI for WM 2617 and VNI/VGII for WM 05.272, WM 05.459, and WM 05.532.

Comparisons of the M13 PCR fingerprints of the putative hybrids with the standard strains of the major molecular types showed that the putative hybrid isolates contained fragments of both proposed parental groups (VNI and VGI for WM 2617 and VNI, and VGII for WM 05.272, WM 05.459, and WM 05.532) as determined by *URA5*-RFLP (Fig. 1 b; Table 1).

After 5 days of incubation, the positive control, *C. gattii* (WM 178), produced a cobalt blue color on CGB medium, whereas the negative control, *C. neoformans* (WM 148), remained yellow (no growth). It is of interest that the three AB hybrids of VNI and VGII produced an intermediate greenish-blue color at day 5, which became blue on day 15, whereas the VNI/VGI hybrid (WM 2617) remained yellow on CGB medium. All four putative hybrids containing serotype B were confirmed by *C. gattii*-specific PCR, giving a positive amplification (data not shown).

Restriction enzyme analysis of the *CAP59* gene with *Bsm*FI, *Hpa*II, and *Age*I showed that all hybrids had both serotype A- and B-specific alleles (Fig. 3). Positive amplifications were obtained for all hybrid isolates with the *MAT*α serotype A allele primer sets specific for *STE20*α (JOHE7264/LOHE7266) and *SXI1*α (JOHE15634/JOHE15635), and the serotype A allele primer sets specific for *GPA1* (JOHE2596/JOHE3241) and *PAK1* (JOHE3066/JOHE3236). No amplification was obtained with the *MAT***a** serotype A allele primer sets specific for *STE20**a*** (JOHE7270/JOHE7271). Similar negative results were obtained when using the primer sets for *SXI2**a*** (JOHE15629/JOHE15630), and serotype D allele primer sets for *GPA1* (JOHE2596/JOHE3240) and *PAK1* (JOHE3066/JOHE3065). In addition, the hybrid isolates yielded amplicons with the primer sets specific for *MF*α (MFαF/MFαR and MFαU/MFαL) and *STE12*α (STE12αF809/STE12αR1607) in *MAT*α and *STE20a* (JOHE9421/JOHE9422) in *MAT***a**. When the STE12aF537/STE12aR1299 primers were used, amplicons were obtained for *MAT***a** in isolates WM 2617 and WM 05.272. The primer set JOHE9787/JOHE9788 amplified *MAT***a** of WM 05.272, whereas MFaF/MFaR primers failed to amplify the hybrid isolates. All the reference strains gave amplicons with the expected primer pairs (Table 2). These results indicate that the hybrid isolates had Aα**B**a combination.

## Discussion

Although *C. neoformans* and *C. gattii* are predominantly haploid organisms, naturally occurring hybrids

**Table 1** Hybrid strains identified in this study

| Strain # | Origin | Source | Date of isolation | Underlying disease | Site of sample | Molecular type | Mating/ serotype |
|---|---|---|---|---|---|---|---|
| WM 2617 | Vellore, Tamil Nadu, India | Clinical | 8/09/1995 | HIV+ | CSF | VNI/VGI | αAB**a** |
| WM 05.272 | Cúcuta, Colombia | Clinical | 2003 | HIV− | CSF | VNI/VGII | αAB**a** |
| WM 05.459 | Castelo do Piaui, Piaui, Brazil | Clinical | 12/1996 | HIV− | CSF | VNI/VGII | αAB**a** |
| WM 05.532 | Caxias, Maranhão, Brazil | Clinical | 03/2001 | HIV− | CSF | VNI/VGII | αAB**a** |

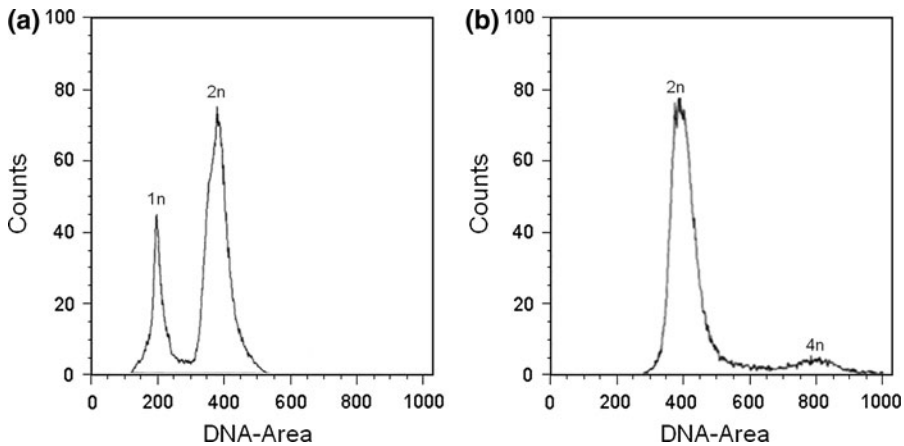


**Fig. 2** Flow cytometry profiles of haploid control strain (**a**) and a putative hybrid isolate (**b**) after staining with the fluorescent dye propidium iodide. *1n*, *2n*, and *4n* indicate nuclear content. The *x*-axis indicates fluorescence intensity reflecting the DNA content, and the *y*-axis indicates cell counts

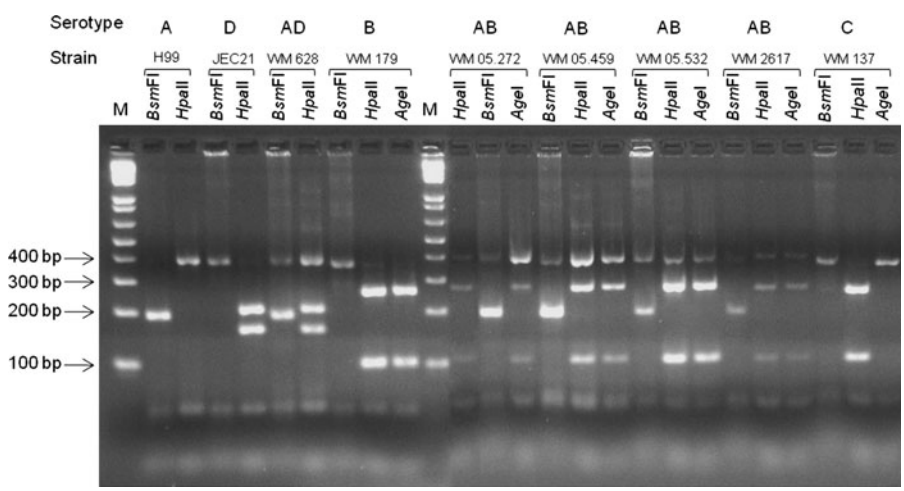


**Fig. 3** Restriction profiles of *CAP59* gene for hybrid isolates and reference strains of different serotypes of *C. neoformans* and *C. gattii* on 3% agarose gel with *Bsm*FI, *Hpa*II, and *Age*I restriction enzymes. *Lane M*, 1-kb$^{+}$ DNA ladder (Invitrogen, Carlsbad, USA)

of AD (inter-varietal), AA (intra-varietal), and BD and AB (inter-species hybrids) that are virulent (i.e., cause mammalian disease) have been reported [1, 24, 25, 27].

In the current study, four AB hybrid strains from patients with cryptococcal meningitis were identified and characterized. By fluorescence-activated cell sorter (FACS) analysis, these strains were diploid or aneuploid. PCR analysis revealed that these strains were heterozygous for serotype A- and B-specific alleles and the *MAT*α and *MAT***a** mating-type loci. Based on these observations, these hybrids could either be produced by somatic fusion followed by karyogamy of a *MAT*α serotype A cell with a *MAT***a** of a serotype B cell, or by mating of a *MAT*α serotype A

**Table 2** Serotype- and mating-type-specific PCR analysis results for the identified hybrid isolates in comparison with reference strains

| Trait | Gene | Isolate/strain | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | WM 2617 | WM 05.272 | WM 05.459 | WM 05.532 | H99 (Aα) | IUM 96-2828 (A**a**) | JEC21 (Dα) | JEC20 (D**a**) | WM 179 (Bα) | WM 04.79 (VG **a**) |
| Genes in the *MAT* locus | *STE20A*α | + | + | + | + | + | − | − | − | ND | ND |
| | *SXI1A*α | + | + | + | + | + | − | − | − | ND | ND |
| | *STE20D*α | − | − | − | − | − | − | + | − | ND | ND |
| | *SXI1D*α | − | − | − | − | – | − | + | − | ND | ND |
| | *STE20A***a** | − | – | − | − | − | + | − | − | ND | ND |
| | *SXI2A***a** | − | − | – | − | − | + | − | − | ND | ND |
| | *STE20D***a** | – | − | − | − | − | − | − | + | ND | ND |
| | *SXI2D***a** | − | − | − | − | − | − | − | + | ND | ND |
| | *MF*α | + | + | + | + | + | − | + | – | + | − |
| | *MF***a** | − | + | − | − | – | + | − | + | − | + |
| | *STE12*α | + | + | + | + | + | _ | ND | ND | ND | ND |
| | *STE12***a** | + | + | − | − | − | + | ND | ND | ND | ND |
| | *STE20***a** | + | + | + | + | ND | ND | ND | ND | − | + |
| Other genes | *GPA1*-A | + | + | + | + | + | + | − | − | ND | ND |
| | *PAK1*-A | + | + | + | + | + | + | − | − | ND | ND |
| | *GPA1*-D | − | − | − | − | − | − | + | + | ND | ND |
| | *PAK1*-D | − | − | − | − | − | − | + | + | ND | ND |

*ND* not done

strain with a *MAT***a** serotype B strain, to produce diploid basidiospores. Different analyses revealed that the strain WM 2617 from India portrayed genetic traits of *C. neoformans* var. *grubii* (molecular type VNI) and *C. gattii* (molecular type VGI), whereas the other three strains (WM 05.272, WM 05.459, and WM 05.532) from South America consisted of a novel genetic hybrid of *C. neoformans* var. *grubii* (molecular type VNI) and *C. gattii* (molecular type VGII). To our knowledge, this is the first report in the literature documenting the occurrence of novel natural hybrids between *C. neoformans* molecular type VNI and *C. gattii* molecular type VGII. It is notable that the strain WM 2617 reacts negative on CGB medium. Negative, weak, and delayed positive reactions on CGB medium have also been shown for other *C. neoformans* × *C. gattii* hybrids [24, 25]. Negative reaction on CGB media may be due to inhibitory effect by canavanine, which is a dominant trait. As such routine screening of isolates on CGB medium may miss those hybrids.

The lack of amplification of the *MAT***a** locus with primer pairs: STE12aF537/STE12aR1299 and JOHE9787/JOHE9788 in some, and of MFaF/MFaR primers in all reported hybrids could be due to a loss of the *MAT***a** allele in some genes of the *C. gattii* parent mating locus during the meiotic process. Alternatively, the primers may not have been specific, due to diverging sequences in the mating-type locus, which prevented annealing of the primers, and hence hindered the amplification, or these isolates were homozygous for one or more mating loci. Loss of genetic material has been observed in AD, BD, and other AB hybrids [24, 25, 45]. Even if hybrid strains possess two genome copies, it is likely that most of the duplicated genes would eventually be lost. However, the presence of two copies of one gene provides an opportunity to evolve new functions, as has been shown for *Saccharomyces cerevisiae* [46].

Development of polyploidy enables tolerance to genetic alteration and provides an additional mechanism for generating genetic diversity. It is a driving force in the evolution of many eukaryotes, and populations with both haploids and diploids may afford greater adaptability than those limited to only one, due to the shuttling between diploid and haploid

states [27]. Since *C. neoformans* and *C. gattii* are environmental pathogens that live in a wide range of ecological habitats, it has been suggested that they have evolved more diverse genotypes and phenotypes to survive in these different niches [47]. Formation of hybrids within *C. neoformans* and *C. gattii* populations might generate genotypic and phenotypic diversity and lineages that can occupy new ecological niches. In this case, hybridization would be advantageous for the fungus. However, it can also be a disadvantage, since some interspecies hybrids exhibit reduced fitness [48]. It has also been observed that diploid strains of *C. neoformans* can convert to a haploid (normal) state, suggesting that polyploidy may be associated with genetic instability [27].

The novelty of the work described herein is that the *C. gattii* parent of three of the four AB hybrids belongs to the VGII molecular type, which has not been reported previously. The *C. gattii* parent of previously reported BD and AB hybrids was of the molecular type VGI [24, 25], which is the same as the hybrid isolate WM 2617 reported in this study. The fact that we found similar hybrids in different countries suggests that either the inter-species mating process is not restricted to a specific geographic area and has independent, non-clonal origins, or that hybrid strains expanded clonally and were dispersed to diverse geographic regions.

It is notable that most hybrids to date have been clinical strains, though the implication of this observation with respect to disease pathogenesis, virulence, and the spread of infection, is not known. Further investigations to determine the relative virulence of these strains are underway.

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