UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Estudo da variabilidade e diferenças morfológicas entre as espécies *Cryptococcus neoformans* e *Cryptococcus gattii* por análise de diferença representacional e microscopia eletrônica de varredura

Tese de Doutorado

Josiane Faganello

Porto Alegre, 2008

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do grau de Doutor em Ciências.

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SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

AIDS	Síndrome da imunodeficiência adquirida
AFLP	Polimorfismo de tamanho de fragmentos amplificados
cDNA	Ácido desoxirribonucléico complementar
^{o}C	Graus Celsius
DNA	Ácido desoxirribonucléico
ESTs	*Expressed sequence tags* (marcadores de seqüências expressas)
GXM	Glicuronoxilomanana
HIV	Vírus da imunodeficiência humana
IGS	Espaçador intergênico
ITS	Espaçador interno transcrito
LCR	Líquido cefalorraquidiano
L-DOPA	3,4-di-hidroxifenilalanina
MATa	*Mating type* a
MATα	*Mating type* alfa
Mb	Megabases
MEV	Microscopia eletrônica de varredura
mRNA	Ácido ribonucléico mensageiro
µL	Microlitro
nt	Nucleotídeos
pb	Pares de bases
PCR	Reação em cadeia da polimerase
pH	Potencial hidrogeniônico
RAPD	DNA polimórfico amplificado aleatoriamente
RDA	Análise de diferença representacional

RFLP	Polimorfismo de tamanho de fragmentos de restrição
SNC	Sistema nervoso central
UTR	Região não traduzida
UV	Ultravioleta
var.	Variedade

RESUMO

Cryptococcus neoformans e *Cryptococcus gattii* são leveduras do grupo dos basidiomicetos que causam criptococcose em indivíduos imunocomprometidos e imunocompetentes, respectivamente. Ambas as espécies são caracterizadas por diferenças moleculares, imunológicas, fisiológicas e epidemiológicas.

Visando identificar diferenças morfológicas, foi desenvolvido um procedimento de preparação alternativo para análise de *C. neoformans* e *C. gattii* por MEV (Microscopia eletrônica de varredura) fixando as células diretamente na cultura em ágar. Este método é mais simples do que os outros já publicados e a morfologia das células foi bem preservada.

Neste trabalho também foi realizado o método de RDA (Análise de diferença representacional) com o objetivo de isolar seqüências que representam diferenças no DNA genômico de *C. neoformans* var. *grubii* e *C. gattii*. Aproximadamente 200 clones foram seqüenciados permitindo a identificação de 19 seqüências diferentes com significante similaridade (Evalue < 10^{-5}) com o genoma completamente seqüenciado de *C. neoformans* var. *neoformans* linhagem JEC21. A maioria das seqüências identificadas representa proteínas hipotéticas ou proteínas de função desconhecida. Experimentos de *Southern blot* com cinco clones selecionados confirmaram a presença de polimorfismos ou especificidade para *C. gattii*. Os dados de seqüenciamento de uma das regiões identificadas como polimórficas, *IDE* (do inglês, *insulin degrading enzime*), foram usados juntamente com os dados de seqüenciamento de outros 3 *loci* (*ACT1, URA5, PLB1*) para estudar as relações filogenéticas entre as espécies. O *locus IDE* mostrou-se mais conservado entre diferentes tipos moleculares do que os outros *loci* estudados, e as variações intra-variedades em *C. gattii* são maiores do que em *C. neoformans*. Estes resultados sugerem que o RDA é um método eficiente para isolar regiões polimórficas de

leveduras e suportam o conceito de duas espécies reconhecido atualmente para o complexo *C. neoformans*.

ABSTRACT

Cryptococcus neoformans and *Cryptococcus gattii* are basidiomycetous yeasts that cause cryptococcosis in immunecompromised and immunecompetent individuals. Both species are characterized by biochemical, immunological, molecular and epidemiological differences.

Aiming to identify morphological differences, we propose an alternative to conventional preparation procedures for scanning electron microscopy (SEM) analysis of *C.neoformans* and *C. gattii* was done fixing the cells directly in the agar culture. This method is simpler than others already reported and the morphology of the cells was well preserved.

We also applied representational difference analysis (RDA) to isolate sequences representing genomic differences between *C. neoformans* var. *grubii* and *C. gattii*. Approximately 200 clones were sequenced leading to the identification of 19 different sequences with significant similarities (Evalue< 10^{-5}) to the completely sequenced genome of the *C. neoformans* var. *neoformans* JEC21 strain. Most of the identified sequences represent hypothetical proteins or proteins of unknown function. Southern blot experiments using five selected clones confirmed the presence of polymorphisms or specificity to *C. gattii*. The polymorphic IDE (insulin degrading enzyme, putative) sequence data were used together with the DNA sequence data of other 3 loci (*ACT1, URA5, PLB1*) for studying phylogenetic relationship between species. The IDE locus was exceptionally conserved among individual molecular types compared with other loci, and intra-varieties variations in *C. gattii* is higher than in *C. neoformans*. Our results suggest that RDA provides an efficient way to isolate polymorphic regions from yeasts and highly supports the two species concept recognized currently.

1. JUSTIFICATIVA

O complexo de espécies *Cryptococcus neoformans*, agente da criptococose, tem sido alvo de muitos estudos há mais de um século. A incidência de criptococose aumentou drasticamente nos últimos anos em função do crescimento da população de indivíduos imunossuprimidos por diferentes fatores, mas principalmente devido à AIDS (Síndrome da Imunodeficiência Adquirida).

A levedura *C. neoformans* foi considerada como uma única espécie homogênea até 1949, quando a existência de quatro sorotipos foi revelada baseada nas características antigênicas de sua cápsula polissacarídica. Por volta dos anos 70, a descrição das fases teleomórficas de *C. neofomans* (*Filobasidiella neoformans* e *Filobasidiella bacillispora*) levou à separação das variedades desta espécie (var. *grubii*, var. *neoformans* e var. *gattii*) em função dos sorotipos conhecidos (sorotipos A, B e D). Nos últimos anos, em função da utilização de ferramentas de biologia molecular e genética, a discussão a respeito da taxonomia de *C. neoformans* aumentou e diversos estudos demonstraram a existência de duas espécies (*C. neoformans* e *C. gattii*) definidas como pertencentes ao complexo *C. neoformans*. Desde então, características epidemiológicas, morfológicas, bioquímicas e genéticas têm sido constantemente estudadas para a melhor caracterização destas espécies.

As espécies do complexo *C. neoformans* têm potencial para se tornarem sistemas modelo para estudos moleculares de doenças fúngicas devido a sua importância clínica, fisiopatologia bem estudada e modelos animais já bem caracterizados. O uso de ferramentas moleculares poderá permitir a identificação

de potenciais alvos para novas drogas antifúngicas e possibilitar o desenvolvimento de vacinas.

Para estudo da morfologia e estrutura capsular, os métodos de microscopia eletrônica, tanto de varredura como de transmissão, foram diversas vezes empregados. Até o momento, a principal técnica utilizada para a visualização de *C. neoformans* por MEV (Microscopia eletrônica de varredura) é de alto custo, laboriosa e envolve a fixação das células em lamínulas cobertas com polilisina antes da desidratação.

A metodologia de RDA (Análise de diferença representacional) já foi aplicada com sucesso diversas vezes para microrganismos procarióticos para a identificação de diferenças genômicas. Até o momento, o método RDA tem sido mais freqüentemente aplicado para o estudo de seqüências diferencialmente expressas em organismos eucarióticos, porém, pouco aplicado para o estudo de diferenças no DNA genômico destes organismos.

Tendo em vista estes aspectos, este trabalho torna-se importante cientificamente ao desenvolver um método prático e eficiente para a visualização e caracterização morfológica de células desta levedura e buscar a identificação de seqüências de DNA específicas ou polimórficas de *C. neoformans* por meio de RDA genômico.

2. REVISÃO BIBLIOGRÁFICA

2.1 O complexo *Cryptococcus neoformans*

A levedura *Cryptococcus neoformans* foi identificada como um patógeno humano em 1894. A primeira descrição de criptococose é geralmente atribuída a dois pesquisadores alemães, Otto Busse e Abraham Buschke, que descreveram o caso de uma mulher com uma lesão na tíbia (CASADEVALL & PERFECT, 1998). O microrganismo foi denominado *Saccharomyces hominis* e a infecção saccharomicosis hominis. Também em 1894, na Itália, Francesco Sanfelice isolou uma levedura encapsulada a partir de suco de pêssego fermentado e, um ano mais tarde, demonstrou a patogenicidade do microrganismo em animais de laboratório. Sanfelice denominou-o *Saccharomyces neoformans* (DROUHET, 1997; CASADEVALL & PERFECT, 1998). Em 1905, Von Hansemann relatou o primeiro caso de meningite criptococócica (MITCHELL & PERFECT, 1995).

Durante vários anos, permaneceram controvérsias relativas à nomenclatura de *C. neoformans*, sendo adotados vários nomes, como *Blastomyces neoformans*, *Cryptococcus hominis* e *Torula histolytica*. Da mesma forma, também a doença teve diferentes denominações, que incluem torulose e blastomicose européia. Relatos de infecção devido a outras espécies do gênero *Cryptococcus* (principalmente *Cryptococcus laurentii* e *Cryptococcus albidus*) são pouco comuns, mas têm aumentado nas últimas décadas, segundo relatos descritos na literatura (MITCHELL & PERFECT, 1995; KHAWCHAROENPORN *et al.*, 2007).

As espécies do complexo *C. neoformans* apresentam antígenos polissacarídicos capsulares que permitem a individualização de cinco sorotipos distintos que foram inicialmente classificados da seguinte forma: os sorotipos A, D e AD, pertencentes à variedade *C. neoformans* var. *neoformans*, e os sorotipos B e C, pertencentes à variedade *C. neoformans* var. *gattii* (MITCHELL & PERFECT, 1995). Entretanto, FRANZOT *et al*. (1999) sugeriram a variedade *grubii* para isolados do sorotipo A, com base nas seqüências do gene URA5, padrões de *DNA fingerprinting* e diferenças fenotípicas. Posteriormente, BOEKHOUT *et al*. (2001) sugeriram a divisão em *C. neoformans* (Sanfelice) Vuillemin e *C. bacillisporus* Kwon-Chung com base em resultados de AFLP (Polimorfismo de tamanho de fragmentos amplificados), distribuição geográfica e origem ecológica. Em 2002, KWON-CHUNG *et al*. (*apud* KWON-CHUNG & VARMA, 2006) também propuseram a divisão em duas espécies: *C. neoformans* e *C. gattii*.

Alguns autores ainda discutem esta divisão, uma vez que *Cryptococcus* pode realizar cruzamentos intervariedades (sorotipo A versus sorotipo D) e interespécies (sorotipo B versus D e sorotipo C versus D) em laboratório e provavelmente na natureza. Porém, a viabilidade dos basidiósporos gerados é reduzida (CASADEVALL & PERFECT, 1998; XU *et al*., 2000; BOVERS *et al*., 2006; LIN & HEITMAN, 2006).

O complexo de espécies também foi subdividido em 9 distintos tipos moleculares com base em polimorfismo de seqüências de DNA detectados por análises utilizando PCR *fingerprinting*, RAPD (DNA polimórfico amplificado aleatoriamente), AFLP, RFLP (Polimorfismo de tamanho de fragmentos de restrição) e MLST (Figura 1) (LIN & HEITMAN, 2006).

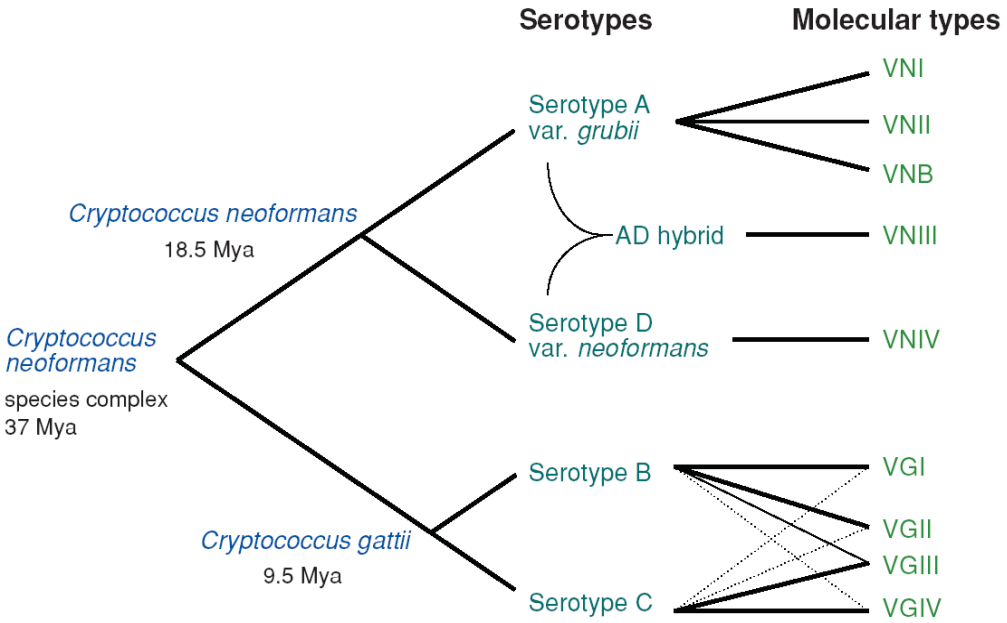

Figura 1. Evolução do complexo de espécies *C. neoformans*. O complexo de espécies Cryptococcus possui pelo menos duas espécies C. neoformans e C. gattii, as quais divergiram de um ancestral comum a 37 e 18,5 milhões de anos, respectivamente. Elas estão divididas em 4 sorotipos consistindo de pelo menos 9 tipos moleculares. As linhas sólidas e tracejadas indicam a prevalência do respectivo sorotipo em cada tipo molecular (Lin & Heitman, 2006).

Isolados do sorotipo AD são híbridos diplóides ou aneuplóides dos sorotipos A e D e contêm alelos típicos de ambos os sorotipos (BOEKHOUT *et al*., 2001; LENGELER *et al*., 2001; XU *et al*., 2003). Embora isolados dos sorotipos A e D tenham sido considerados geneticamente distintos, é possível obter cruzamento entre isolados dos dois sorotipos (KWON-CHUNG, 1975 *apud* SCHEIN *et al*., 2002). A correta classificação dos isolados do sorotipo AD no que diz respeito à classificação em variedades, não está bem resolvida (NISHIKAWA *et al*., 2003).

Em amostras clínicas, *C. neoformans* é quase sempre encontrado na forma de levedura (estágio anamórfico assexuado) e se reproduz por brotamento. O

estágio sexual (perfeito ou teleomórfico) é caracterizado pela presença de basidiosporos e é observado somente durante o cruzamento (*mating*) não tendo ainda sido descrita nenhuma associação com amostras clínicas (CASADEVALL & PERFECT, 1998). Existem dois *mating type*s para *C. neoformans*, α e a, determinados por um único *locus*, sendo o *mating type* α prevalente em isolados clínicos (>99%) e ambientais subtropicais (KWON-CHUNG & BENNETT, 1978; CASALI *et al*., 2003). Sob condições de limitação de nutrientes ocorre a fusão entre *mating type*s opostos e a geração do estágio perfeito, *Filobasidiella neoformans* (Figura 2, painel superior). O estágio perfeito é caracterizado pela formação transitória de uma hifa dicariótica com um grampo de conexão típico de basidiomicetos. Um basídio é formado na porção terminal da hifa onde a cariogamia e a meiose ocorrem com subseqüente formação de cadeias de basidiosporos na superfície do basídio. A germinação dos basidiosporos produz células leveduriformes que caracterizam o estágio assexual ou imperfeito (LIN & HEITMAN, 2006).

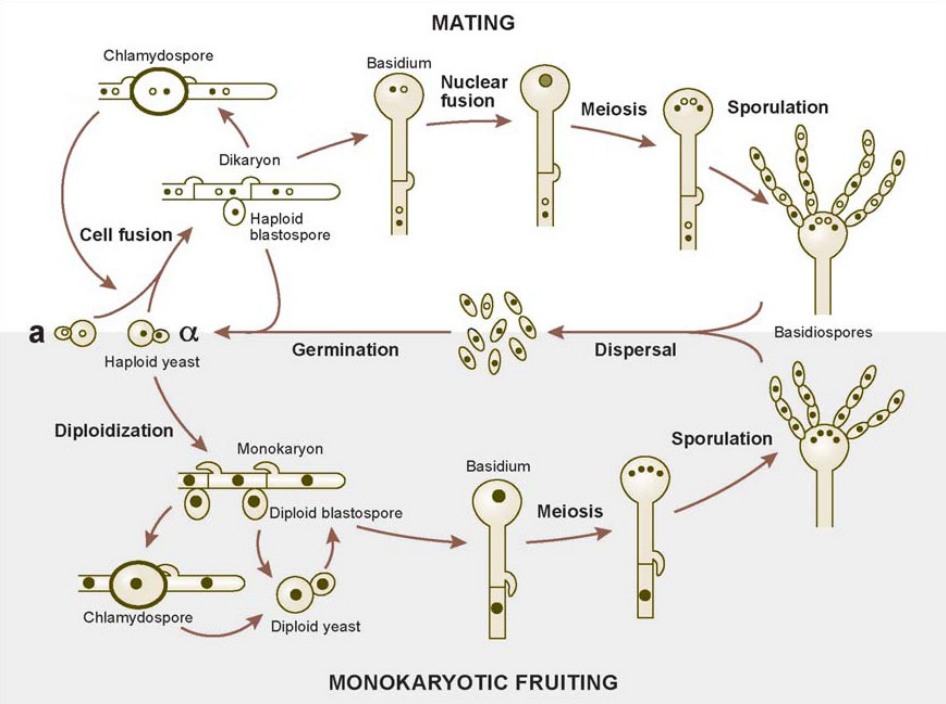

Figura 2. Modelo do ciclo de vida de *C. neoformans*. O painel superior mostra o ciclo sexual tradicional envolvendo parceiros de *mating type* oposto, enquanto o painel inferior mostra a frutificação monocariótica que ocorre entre parceiros do mesmo *mating type* (Lin & Heitman, 2006).

Na ausência de *mating* compatível e sob condições de diminuição de fontes de nitrogênio, células haplóides de *C. neoformans* podem sofrer uma modificação morfológica e produzir hifas com basídio terminal e basidiosporos. Esse fenômeno é conhecido como frutificação haplóide, monocariótica ou homocariótica e é comum em basidiomicetos superiores (Figura 2, painel inferior)(HULL & HEITMAN, 2002; LIN & HEITMAN, 2006). WICKES *et al*. (1996) demonstraram que a frutificação haplóide estaria restrita ao *mating type* α, o que seria uma hipótese atrativa para justificar a preponderância de células MATα na natureza e em amostras clínicas. Porém, posteriormente, foi demonstrado que a frutificação haplóide em *C. neoformans* não é exclusiva de células MATα, podendo também ocorrer em células MATa, e não existindo evidência para

qualquer associação genética entre o *mating type* e a frutificação haplóide (TSCHARKE *et al*., 2003).

Diversas características biológicas desta levedura sugerem populações clonais na natureza. Apesar de existirem dois *mating type*s, a reprodução sexual entre eles foi demonstrada somente sob condições apropriadas, em laboratório. Além disso, a predominância do *mating type* α sugere que a recombinação sexual em populações naturais de *C. neoformans* não ocorreria ou seria pouco freqüente e a grande heterogeneidade no tamanho e número dos cromossomos de isolados de *C. neoformans* sugere incompatibilidade de cruzamento (FRANZOT *et al*., 1997). Evidências de reprodução sexual entre isolados do mesmo *mating* foram encontradas e a ocorrência deste fenômeno foi verificada primeiramente *in vitro* (FRASER *et al*., 2005; LIN *et al*., 2005). Atualmente, já há também fortes evidências de que este tipo de reprodução também ocorre na natureza, o que explica a grande prevalência do *mating type* α em isolados clínicos e ambientais (LIN *et al*., 2007). Estes achados mostram que a reprodução entre isolados de mesmo *mating* pode ser importante para a expansão de patógenos humanos para novos nichos e contribuir para a produção continuada de esporos infecciosos.

2.2 Criptococose e epidemiologia

A criptococose é uma infecção aguda, subaguda ou crônica, causada pelas espécies do complexo *C. neoformans*, que comumente envolve o cérebro, o pulmão, os ossos ou a pele (CASADEVALL & PERFECT, 1998). É considerada a infecção fúngica sistêmica oportunista mais freqüente em pacientes com AIDS,

sendo que aproximadamente 3 milhões de pacientes infectados pelo HIV (Vírus da imunodeficiência humana) vêm a óbito a cada ano em decorrência de complicações associadas a criptococose, de acordo com a Organização Mundial de Saúde e o Programa HIV/AIDS das Nações Unidas (LIN & HEITMAN, 2006).

C. neoformans é um patógeno oportunista, que causa doença invasiva em indivíduos hígidos ou imunocomprometidos. A maioria dos pacientes atingidos tem a função imune comprometida como uma conseqüência de infecção por HIV, terapia com corticosteróides, câncer, quimioterapia ou terapia para diminuir a rejeição de órgãos transplantados. *C. gattii*, por sua vez, é um patógeno primário, que causa infecção em indivíduos hígidos. A meningite é a forma mais usualmente encontrada da doença por estes dois patógenos (CASADEVALL & PERFECT, 1998; DEL VALLE & PINA-OVIEDO, 2006). O arsenal de agentes antifúngicos utilizados é limitado, e os efeitos tóxicos e a emergência de resistência às drogas são impedimentos para uma terapia efetiva.

A incidência da criptococose varia de acordo com a população, a região e o período estudado. No Brasil, de 1980 a 2002, 215.810 pacientes com AIDS foram registrados, sendo que, entre estes, 6% tinham criptococose no momento do diagnóstico (MINISTÉRIO DA SAÚDE, PROGRAMA DST-AIDS - 2002). No Rio Grande do Sul, entre 1995 e 2003, foram registrados 638 casos de meningite criptocócica pela Secretaria de Saúde do Estado. Admite-se, no entanto, que o número real de casos pode ser consideravelmente maior, uma vez que a doença não é de notificação compulsória (ABEGG, 2003).

O mecanismo de infecção de um indivíduo se dá por via respiratória (inalação) e acredita-se que os basidiosporos (produzidos durante a reprodução

sexuada ou frutificação haplóide) ou as células leveduriformes dissecadas representem o propágulo infeccioso. Ambos apresentam tamanho ideal (1-2 μm) para a deposição nos alvéolos pulmonares, quando comparados ao tamanho da célula encapsulada (3-8 μm de diâmetro) (SUKROONGREUNG *et al*., 1998; HALLIDAY & CARTER, 2003). Existem casos de infecção assintomática, com a permanência do microrganismo na forma latente no indivíduo infectado, mas o mais comum é o desenvolvimento de infecção sistêmica seguida de infecções localizadas, como, por exemplo, na pele, e principalmente neurotropismo (LIN & HEITMAN, 2006).

Existem pelo menos três aspectos que poderiam explicar o neurotropismo da infecção por *C. neoformans* e *C. gattii* (LIN & HEITMAN, 2006). Primeiro, a atração da levedura por substratos específicos presentes no SNC (Sistema nervoso central). Segundo, o SNC poderia servir como um refúgio para a levedura proteger-se do sistema imune do hospedeiro. Terceiro, a presença de receptores específicos presentes nas células neuronais poderia atrair mais as células de *C. neoformans* e *C. gattii*, comparado a outros órgãos durante a infecção sistêmica.

O diagnóstico laboratorial pode ser realizado pelo exame do LCR (Líquido cefalorraquidiano) com tinta da Índia, exame histopatológico ou ainda pelo teste de aglutinação em látex para detecção do antígeno polissacarídico capsular. Após o exame do material clínico, faz-se o isolamento em cultura e identificação final por testes bioquímicos, como assimilação de carboidratos, produção de urease e produção de pigmento melanóide quando semeado em meios ricos em compostos fenólicos, como, por exemplo, ágar semente de níger (*Guizotia abyssinica*) ou ágar semente de girassol (*Helianthus annuus*). A ocorrência de

formas subclínicas ou assintomáticas da doença não tem sido propriamente avaliada e estudos, tais como pesquisa de imunidade celular usando testes intradérmicos com o antígeno da levedura em pacientes sem doença clínica, são raros (SIDRIM & MOREIRA, 1999; PAPPALARDO & MELHEM, 2003). Aproximadamente 95% de todas as infecções por leveduras do complexo *C. neoformans* são causadas por isolados do sorotipo A, e mais de 99% das infecções em pacientes com AIDS também são causadas por esse mesmo sorotipo. No Brasil, a maioria dos isolados clínicos e ambientais são do sorotipo A, com exceção das regiões norte e nordeste, onde o sorotipo B é endêmico (CASALI *et al*., 2003; NISHIKAWA *et al*., 2003; ABEGG *et al*., 2006).

C. neoformans e *C. gattii* são fungos cosmopolitas e já foram isolados de uma variedade de fontes ambientais, principalmente de substratos orgânicos e habitats relacionados, excretas de aves, frutos, suco de frutas fermentado, leite, cavernas, água, solo e ocos de várias espécies de árvores (CASADEVALL & PERFECT, 1998; ABBEG *et al*., 2006; MEDEIROS RIBEIRO *et al*., 2006; KIDD *et al*., 2007). *C. neoformans* var. *grubii* tem distribuição mundial; enquanto *C. neoformans* var. *neoformans* é encontrado principalmente no sul da Europa e *C. gattii* em regiões tropicais e subtropicais (CASALI *et al*., 2003). Entretanto, foi descrita uma epidemia de criptococose causada por *C. gattii* em Vancouver, Canadá, região de clima temperado. Desde 1999, mais de 66 casos em indivíduos saudáveis, com pelo menos 6 casos com óbito, foram atribuídos à infecção por *C. gattii* (HOANG *et al*. 2004; FRASER *et al*., 2005; Kidd *et al*., 2007; UPTON *et al*., 2007).

2.3 Fatores de virulência

A patogênese da criptococose envolve vários fatores de virulência, ou seja, fatores que permitem que a levedura se instale no hospedeiro, sobreviva e cause doença. São três os principais fatores de virulência necessários para as espécies do complexo *C. neoformans* estabelecerem infecção no hospedeiro humano: presença de cápsula polissacarídica, capacidade de produzir melanina e capacidade de crescimento a 37°C (PERFECT, 2005).

É importante considerar nos estudos dos fatores de virulência de *C. neoformans* e *C. gattii* o fato desse organismo não requerer um hospedeiro humano para completar o seu ciclo de vida. Assim, os fatores que aumentam a capacidade do fungo em causar doença têm um papel primário de garantir a sobrevivência do organismo na natureza (ALSPAUGH *et al.*, 1998). Tem sido proposto que a interação do fungo com amebas e nematódeos presentes no ambiente permitiu ao patógeno desenvolver estratégias para sobreviver ao sistema imune do hospedeiro (HULL & HEITMAN, 2002; STEENBERGEN & CASADEVALL, 2003).

O fator de virulência melhor caracterizado em *C. neoformans* e *C. gattii* é a cápsula polissacarídica. Ela permite a identificação rápida da levedura sendo a base do método clássico com tinta da Índia em líquido cefalorraquidiano no diagnóstico da meningite criptococócica (ALSPAUGH *et al.*, 1998). Mais de 90% do envelope celular da levedura é composto de carboidratos e o seu constituinte primário é um polissacarídeo denominado GXM (Glicuronoxilomanana). As diferenças na estrutura da GXM entre isolados são responsáveis pela

diferenciação antigênica de *C. neoformans*, resultando na separação dos cinco sorotipos distintos (A, B, C, D e o híbrido AD) (NISHIKAWA *et al*., 2003). Mais recentemente, foram descritos isolados clínicos identificados como híbridos BD. Foram realizadas análises de AFLP e seqüenciamento que indicaram a presença dos genótipos AFLP 2 (*C. neoformans*) e AFLP 4 (*C. gattii*) e, sorologicamente, estas amostras foram caracterizadas como BD (BOVERS *et al*., 2006).

Na natureza, a cápsula pode proteger a levedura da dessecação e reduzir a sua ingestão por amebas presentes no solo (McFADDEN *et al*., 2006). No hospedeiro humano, a presença de cápsula inibe a fagocitose por células do sistema imunológico, ativa a cascata do complemento, inibe a migração de leucócitos, interfere na apresentação de antígenos e desregula a secreção de citocinas (PERFECT, 2005).

C. neoformans e *C. gattii* produzem uma enzima fenoloxidase, identificada como uma lacase, que pode sintetizar melanina a partir de precursores como L- e D-DOPA, dopamina, epinefrina e norepinefrina (EISENMAN *et al*., 2007). A melanina, localizada na parede celular do fungo, pode funcionar como proteção contra radiações UV no ambiente e como fator de virulência no organismo hospedeiro, uma vez que protege a levedura contra os efeitos oxidantes provenientes das células de defesa e pode explicar o neurotropismo deste microrganismo. Além disso, a produção de melanina serve como uma característica de identificação de isolados de *C. neoformans* e *C. gattii* ao tornar as colônias pigmentadas em meios contendo compostos difenólicos. A produção de melanina também pode interferir na suscetibilidade a antifúngicos e proteção

contra temperaturas extremas (BUCHANAN & MURPHY, 1998; STEENBERGEN & CASADEVALL, 2003).

Para o desenvolvimento de *C. neoformans* e *C. gattii* à temperatura de 37°C (temperatura fisiológica humana), é necessária a expressão da subunidade catalítica A da proteína calcineurina (CNA1) (BUCHANAN & MURPHY, 1998; STEINBACH *et al*., 2007). Muitas estratégias têm sido utilizadas para caracterizar genes envolvidos com este fator de virulência. Até o momento, 15 genes já foram identificados como sendo necessários para o desenvolvimento das leveduras a altas temperaturas (PERFECT, 2006). Rosa e Silva *et al*. (2008) também identificaram por RDA produtos gênicos cuja transcrição foi maior à temperatura de 37°C, como, por exemplo, genes envolvidos na manutenção da integridade da parede celular, na resposta a estresse, na filamentação e no metabolismo oxidativo e de ácidos graxos.

Outras características que contribuem para a virulência incluem as a secreção de proteinases pela levedura, produção de urease, secreção de fosfolipases extracelulares, *mating type* α, e produção de manitol. A secreção de proteinases por *C. neoformans* e *C. gattii* tem sido estudada e acredita-se que essas enzimas contribuam para a virulência do microrganismo, degradando proteínas dos tecidos do hospedeiro, como colágeno, elastina e fibrinogênio, e/ou destruindo outras proteínas com função imunológica importante, como imunoglobulinas e fatores do complemento (STEENBERGEN & CASADEVALL, 2003). Também a secreção de urease é considerada um fator de virulência, uma vez que a alcalinização do meio favorece a sobrevivência do fungo no fagolisossoma (COX *et al*., 2000). A atividade da enzima fosfolipase (PLB1) está

associada à virulência devido à destruição de componentes da membrana celular e lise celular no hospedeiro e o gene responsável por esta atividade já foi clonado e caracterizado (COX *et al*., 2001; STEENBERGEN & CASADEVALL, 2003).

Outro fator de virulência, a produção de manitol, um poliálcool acíclico, pode contribuir para a elevação da pressão intracraniana em pacientes com meningoencefalite ao provocar o aumento da pressão osmótica e pode, de forma semelhante à melanina, atuar como um antioxidante (CASADEVALL & PERFECT, 1998).

A predominância do *mating type* α entre os isolados clínicos e ambientais (30 a 40 vezes mais que o *mating type* a) e a evidência de que células MATα são mais virulentas em camundongos, sugere que o *mating type* α funcione como um fator de virulência (KWON-CHUNG *et al*., 1992 *apud* HEITMAN, 2006). Porém, embora o *locus* de *mating type* já tenha sido clonado, o mecanismo, os genes, ou os produtos destes genes pelos quais o *mating type* afeta a virulência permanecem desconhecidos (STEENBERGEN & CASADEVALL, 2003; MITCHELL *et al*., 2003). As diferenças genéticas existentes entre os dois *mating type*s podem ser a causa de uma maior adaptação de um em relação ao outro tanto na natureza como no hospedeiro, explicando assim a prevalência do *mating type* α entre isolados clínicos (MCCLELLAND *et al*., 2004).

2.4 Microscopia eletrônica de varredura

As células de *C. neoformans* e *C. gattii* são típicas células leveduriformes, esféricas a ovais. Apresentam tamanho variável, contorno duplo e podem ser

altamente refráteis. O método mais comum para a visualização das células emprega uma suspensão diluída de tinta da Índia. Como as partículas de tinta da Índia são excluídas pela cápsula polissacarídica, esta aparece como uma área clara em torno da célula (CASADEVALL & PERFECT, 1998). Uma modificação desta técnica, utilizando mercúrio cromo, permite a visualização de estruturas internas da levedura através da microscopia óptica (ZERPA *et al*., 1996).

Existem algumas características morfológicas já descritas que podem ser associadas a cada uma das espécies (KWON-CHUNG *et al*., 1982 *apud* KWON-CHUNG & VARMA, 2006). As colônias de *C. gattii* em meio de cultivo são quase sempre mais mucóides do que as de *C. neoformans*, embora isso nem sempre reflita no tamanho da cápsula polissacarídica. As células de *C. gattii* são mais freqüentemente reportadas como sendo elípticas, enquanto esta característica é pouco freqüente em *C. neoformans*.

A microscopia eletrônica deste fungo vem sendo realizada ao longo dos anos e mostra que *C. neoformans* e *C. gattii* têm características típicas de células eucarióticas. Já foram descritos detalhes da cápsula e das estruturas internas da célula com base em fotomicrografias de secções finas observadas em microscopia de transmissão (EDWARDS *et al*., 1967; CASADEVALL & PERFECT, 1998).

Vários estudos utilizando microscopia eletrônica avaliaram a produção de melanina a partir de L-dopa (3,4-di-hidroxifenilalanina) com aumento da densidade da parede celular (WANG *et al*., 1996; NOSANCHUK & CASADEVALL, 2003). A MEV tem sido útil na investigação de características dos polissacarídeos capsulares (CLEARE & CASADEVALL, 1999), formação de brotamentos em

células melaninizadas (NOSANCHUK & CASADEVALL, 2003), bem como na avaliação dos efeitos de concentrações subinibitórias dos antifúngicos anfotericina B e fluconazol na morfologia celular de *C. neoformans* e *C. gattii* (NOSANCHUK *et al*., 1999). Mais recentemente, a MEV foi aplicada para investigar a formação de biofilmes por *C. neoformans* e *C. gattii* em diferentes suportes e a suscetibilidade destes biofilmes a condições de estresse como luz UV e calor (MARTINEZ & CASADEVALL, 2007).

2.5 Biologia molecular de *C. neoformans* e *C. gattii*

Assim como tantas diferenças fenotípicas existentes entre as variedades do complexo de espécies *C. neoformans*, também as diferenças genotípicas têm sido estudadas, principalmente a partir da década de 80 (CASADEVALL & PERFECT, 1998). Vários sistemas de tipificação molecular têm sido desenvolvidos e utilizados para *C. neoformans* e *C. gattii*. Entre eles podemos citar a análise dos padrões de fragmentos de DNA gerados por RFLPs (FAN *et al*., 1994), o *DNA fingerprinting*, por hibridização de fragmentos gerados por enzimas de restrição com sondas de DNA genômico repetitivo (CHEN *et al*., 1995; SPITZER & SPITZER, 1997), cariótipo eletroforético por eletroforese de campo pulsado (FRANZOT *et al*., 1997), métodos de PCR *fingerprinting* (SORRELL *et al*., 1996; MEYER *et al*., 1999), a análise por RAPD (CHEN *et al*., 1996; HORTA *et al*., 2002), tipagem com enzima multilocus (BRANDT *et al*., 1993), seqüenciamento das regiões IGS (Espaçador intergênico) e ITS (Espaçador interno transcrito) (DIAZ *et al*., 2000; KATSU *et al*., 2004) e, mais recentemente,

multilocus sequence typing (MLST) (LITVINTSEVA *et al*., 2006; BOVERS *et al*., 2008).

Na década de 90, dois diferentes sistemas de transformação foram desenvolvidos para *C. neoformans* e *C. gattii*. No primeiro, o DNA pode ser introduzido por eletroporação. No segundo, o DNA é introduzido por biolística utilizando microprojéteis de ouro transportando o DNA. Estes estudos permitiram a possibilidade de análise da função de genes por transformação e interrupção de genes por integração homóloga (HULL & HEITMAN, 2002). Posteriormente, a transformação mediada pela bactéria *Agrobacterium tumefaciens*, descrita para *C. neoformans* em 2002, foi otimizada e, quando comparada aos outros métodos, apresentou eficiência semelhante para *C. neoformans* e superior para *C. gattii* (MCCLELLAND *et al*., 2005).

Foi publicado por LOFTUS *et al*. (2005) o relato do seqüenciamento do genoma de isolados de referência de *C. neoformans* var. *neoformans*, JEC21 e B3501 (ambos pertencentes ao sorotipo D). Outros genomas atualmente disponíveis pertencem ao isolado patogênico de *C. neoformans* var. *grubii* H99 (sorotipo A) e duas linhagens de *C. gattii*, WM276 e R265 (ambas do sorotipo B, sendo W276 um isolado ambiental e R265 um isolado clínico relacionado à epidemia de Vancouver) (HOANG *et al*. 2004; LIN & HEITMAN, 2006).

C. neoformans possui um genoma de aproximadamente 19 Mb de DNA, em 14 cromossomos que variam em tamanho (762 kb a 2,3 kb), e 6572 genes ricos em íntrons (uma média de 5,3 íntrons por gene com tamanho médio de 67 pb) (LOFTUS *et al*., 2005). Aproximadamente 50% das seqüências dos genomas do isolado B-3501 e do isolado JEC21 são idênticas, e o restante das seqüências

apresentam pequenos polimorfismos. Cerca de 5% do genoma de *Cryptococcus* é composta por transposons, que se agrupam em blocos únicos nos cromossomos, variando de 40 a 100 kb.

A organização gênica em *C. neoformans* e *C. gattii* é consideravelmente mais complexa do que a dos ascomicetos cuja seqüência está disponível e é comparável àquela observada em *Arabidopsis thaliana* ou *Caenorhabditis elegans*. *Splicing* alternativo foi predito para 277 genes, ou 4,2% do transcriptoma, e uma variedade de mecanismos de *splicing* foi identificada. A análise do genoma identificou mais de 30 genes provavelmente envolvidos na biosíntese de cápsula. Sessenta e cinco por cento dos genes de *C. neoformans* têm homologia conservada com outros genomas de fungos, 10% parece ser único de *C. neoformans*, enquanto os 25% restantes não têm identidade com qualquer seqüência de fungos disponível em bancos de dados (LOFTUS *et al*., 2005).

O genoma deste fungo é particularmente rico em elementos repetitivos, o que é pouco usual para um microrganismo e mais típico de eucariotos superiores. A expansão de elementos repetitivos num genoma poderia produzir eventos de recombinação aberrantes e instabilidade do genoma, causando mudanças em nível genômico tais como duplicações, deleções, translocações, inversões e mutações (LIN & HEITMAN, 2006). Posteriormente, ROY *et al*. (2007) estudaram íntrons de regiões não traduzidas (UTRs) das espécies do complexo *C. neoformans* e observaram que o tamanho médio e densidade média dos íntrons nas espécies estudadas é menor do que os demais padrões relatados de plantas e animais. Também baseados em dados de seqüenciamento dos genomas de *Cryptococcus*, SHARPTON *et al*. (2008) estudaram a taxa de perda de íntrons e

descreveram um gene (CNN00420) que ganhou um intron por meio da duplicação de DNA repetitivo.

Como *C. neoformans* e *C. gattii* geram e mantêm o polimorfismo genético e fenotípico e como seu modo de vida e reprodução no ambiente afeta sua evolução ainda é pouco compreendido. Microevolução nestas espécies pode resultar em mudanças fenotípicas e genotípicas neste organismo e mudanças de fenótipo (*phenotypic switching*) já foram caracterizadas em *C. neoformans* e *C. gattii* (JAIN *et al*., 2006).

Estudos de filogenética molecular demonstraram que os sorotipos do complexo *C. neoformans* divergiram uns dos outros há milhões de anos. As variedades *grubii* e *neoformans* são separadas por aproximadamente 18 milhões de anos de evolução, e essas variedades diferem de *C. gattii* por aproximadamente 37 milhões de anos (XU *et al*., 2000). Mais recentemente, utilizando-se os dados dos genomas de *Cryptococcus* disponíveis, estimou-se que as duas espécies divergiram há 80 milhões de anos (SHARPTON *et al*., 2008).

2.6 Análise de diferença representacional

O método de RDA, desenvolvido por LISITSYN *et al*. (1993), baseia-se em hibridização subtrativa e enriquecimento cinético, purificando fragmentos presentes em uma população de fragmentos de DNA que estejam ausentes em outra população. Desta forma, o RDA é um método desenvolvido para a análise de pequenas diferenças existentes entre as seqüências de duas populações de

DNA, tornando-se útil para a descoberta de agentes infecciosos e sondas para estudos genéticos.

A hibridização subtrativa clássica envolve a hibridização de DNA de uma das amostras (*tester*) com grande excesso de outra amostra de DNA (*driver*), de forma que os fragmentos de DNA *tester* formem híbridos predominantemente com os fragmentos do DNA *driver* (LISITSYN, 1995). A hibridização é geralmente seguida por uma separação física das seqüências indesejadas da seqüência-alvo, usando, por exemplo, cromatografia. Isso resulta na purificação dos fragmentos de DNA-alvo que estão presentes somente na amostra de DNA *tester*. Contudo, quanto maiores e mais complexos os genomas ou quanto menor a diferença entre eles, mais difícil seria essa separação. Por isso a hibridização subtrativa foi considerada ineficiente para a comparação de DNA genômico de eucariotos superiores. A hibridização subtrativa bem sucedida geralmente resulta em apenas um enriquecimento de aproximadamente 100 a 1000 vezes da seqüência alvo e, portanto, deve ser seguida por um processo de classificação através de um grande número de seqüências de DNA para encontrar uma de interesse (LISITSYN, 1995).

No método de RDA a complexidade dos genomas foi reduzida através da preparação de porções representativas de cada genoma (“representações”). A reduzida complexidade das representações nos permite alcançar um maior rendimento durante a etapa de hibridização subtrativa e, conseqüentemente, um enriquecimento cinético mais efetivo (LISITSYN, 1995). Portanto, torna-se importante a escolha da enzima de restrição adequada para gerar fragmentos de DNA de tamanho adequado. Se uma população de fragmentos de DNA contendo

uma subpopulação-alvo enriquecida “n” vezes em relação aos fragmentos não enriquecidos no *tester* é fundida e reanelada de maneira que somente uma pequena proporção do DNA *tester* dupla-fita se forme, o DNA alvo dupla-fita poderia estar presente “n^2” vezes em relação às outras seqüências presentes como DNA dupla-fita. Isso é denominado enriquecimento cinético (LISITSYN *et al*., 1993).

O RDA aplica este princípio por amplificação do DNA por PCR (Reação em cadeia da polimerase) para purificar as pequenas quantidades de DNA dupla-fita de *tester*. Para amplificar os pequenos fragmentos de restrição, um oligonucleotídeo adaptador contendo uma seqüência *primer* para a PCR é ligado em ambas as pontas de todos os fragmentos do *tester*, de forma que somente as moléculas dupla-fita do tester sejam amplificadas (LISITSYN *et al*., 1993). Uma combinação de adaptadores é usada e consiste dos *primers* desejados para a PCR, um com 24 bases e outro com 12 bases. Este último é complementar ao oligonucleotídeo de 24 bases e ao sítio de restrição. O anelamento lento cria uma ponte temporária formada pelos 12 nucleotídeos que posicionam os 24 nucleotídeos adjacentes ao fragmento de DNA digerido, permitindo a ligação direta do *primer* (24 nt) na extremidade 5’ do fragmento de DNA. Posteriormente, o amplicon é purificado e a Taq DNA polimerase pode gerar fitas duplas do *primer* requerido para a amplificação exponencial por PCR (PASTORIAN *et al*., 2000).

Entre as aplicações inicialmente sugeridas para a análise de diferença representacional estão a detecção de anormalidades genéticas que resultam em câncer, geração de marcadores de RFLPs que existem entre espécies ou indivíduos relacionados para serem usados no mapeamento genético e a

identificação de *loci* ligados a doenças genéticas que resultam de mutações espontâneas ou rearranjos no óvulo fertilizado (LISITSYN *et al*., 1993). Desta forma, este método pode ser muito útil na geração de sondas para estudos genéticos.

Embora o RDA, na sua forma original, tenha sido projetado para identificar diferenças entre populações de DNA, o mesmo processo foi subseqüentemente modificado por HUBANK & SCHATZ (1994) para permitir a análise de diferenças em populações expressas de RNA mensageiro (mRNA), através do cDNA (DNA complementar) correspondente. Essa técnica, cDNA – RDA, permite a detecção de mudanças na expressão de mRNAs por enriquecimento seletivo sem qualquer conhecimento prévio da seqüência do gene específico em questão. Esta seria a vantagem frente aos métodos clássicos cujo fator limitante para análise dos padrões de expressão é a aplicabilidade somente para genes conhecidos (PASTORIAN *et al*., 2000).

Com relação ao estudo de bactérias de importância clínica humana e veterinária, esta técnica já se mostrou útil, por exemplo, para a identificação de seqüências específicas de *Brachyspira hyodysenteriae*, agente etiológico da disenteria suína (ROTHKAMP *et al*., 2002) e seqüências específicas de *Escherichia coli* sorotipo O157, importante patógeno associado à diarréia humana grave (ALLEN *et al*., 2001), além de trabalhos envolvendo outras bactérias como *Pseudomonas aeruginosa* (CHOI *et al*., 2002), *Yersinia enterocolitica* (IWOBI *et al*., 2002), *Vibrio cholerae* (CALIA *et al*., 1998) e *Neisseria meningitidis* (BART *et al*., 2000; BART *et al*., 2001).

Em relação a organismos eucariotos inferiores, a metodologia de RDA genômico foi empregada, por exemplo, para a identificação de seqüências específicas do DNA da fêmea de *Schistosoma mansoni* (DREW & BRINDLEY, 1995). O emprego da metodologia de cDNA-RDA para *Leishmania* (Viannia) *panamensis* permitiu a clonagem do gene histona H1 (ALZATE *et al*., 2006). Da mesma forma, esta metodologia possibilitou a clonagem de genes expressos durante a formação do corpo de frutificação no basidiomiceto *Lentinula edodes* (MIYAZAKI *et al*., 2005).

DUTRA *et al*. (2004) com a finalidade de encontrar genes diferencialmente expressos pelo fungo *Metarhizium anisopliae* durante o processo de infecção do carrapato *Boophilus microplus*, utilizou a metodologia de RDA de cDNA, comparando o cDNA gerado em meio com cutícula do carrapato e cDNA gerado em meio rico em glicose. Entre 135 clones seqüenciados, foram identificados, após comparação com bancos de dados, 34 seqüências e 14 ESTs (*Expressed sequence tags*) com ortólogos conhecidos. Mais recentemente, BAILÃO *et al*. (2007) estudaram genes diferencialmente expressos em condições de interação do patógeno *Paracoccidiodes brasiliensis* com o hospedeiro e BAEZA *et al*. (2007) utilizaram a metodologia de cDNA-RDA para a identificação de genes diferencialmente expressos por *Trichophyton rubrum* em presença de queratina.

Para *C. neoformans*, o perfil de expressão gênica foi avaliado por cDNA RDA permitindo a identificação de novos genes regulados positivamente em temperatura de 37°C, possivelmente relacionados com a interação patógeno-hospedeiro (ROSA E SILVA *et al*., 2008). Os produtos destes genes estão envolvidos em diferentes processos biológicos, como, por exemplo, na integridade

da parede celular, resposta a estresse e metabolismo oxidativo. Neste estudo também observou-se que genes relacionados ao transporte de fosfolipídios e estrutura da cromatina foram regulados positivamente em temperatura de 25℃.

3. OBJETIVOS

3.1 Geral

Identificar diferenças morfológicas e caracterizar seqüências de DNA que possam auxiliar na determinação da variabilidade de isolados clínicos e ambientais de *C. gattii* e das variedades *grubii* e *neoformans* de *C. neoformans*.

3.2 Específicos

- Identificar seqüências possivelmente específicas ou polimórficas entre *C. gattii* e *C. neoformans* var. *grubii* utilizando a metodologia de RDA;
- Adaptar a metodologia de MEV para utilização direta a partir de meio de cultura;
- Comparar a morfologia celular de *C. gattii* e *C. neoformans* var. *grubii* por MEV utilizando a metodologia desenvolvida.

4. RESULTADOS

4.1 Análise de diferenças genômicas e análise filogenética

MANUSCRITOS SUBMETIDOS

Medical Mycology

4.1.1. Identification of genomic differences between Cryptococcus neoformans and Cryptococcus gattii by a RDA approach

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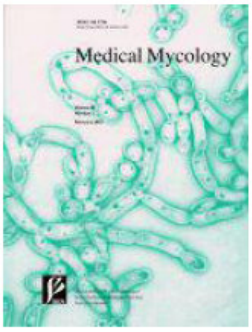

Identification of genomic differences between Cryptococcus neoformans and Cryptococcus gattii by a RDA approach

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Identification of genomic differences between *Cryptococcus neoformans* and *Cryptococcus gattii* by a RDA approach

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SUMMARY

Cryptococcus neoformans and *Cryptococcus gattii* are basidiomycetous yeasts that cause cryptococcosis in immunocompromised and immunocompetent individuals. In this study, Representational Difference Analysis (RDA) was applied to isolate sequences representing genomic differences between *C. neoformans* var. *grubii* and *C. gattii*. Approximately 200 clones were sequenced leading to the identification of 19 different sequences with significant similarities ($E_{value}< 10^{-5}$) to the completely sequenced genome of the *C. neoformans* var. *neoformans* JEC21 strain. Southern blot experiments using five selected clones confirmed the presence of polymorphisms for three sequences (a putative Insulin Degrading Enzyme (*IDE*), a chitin synthase and an endoplasmic reticulum protein). The results demonstrated that this methodology, when properly performed, can be successfully applied for the identification of differences between two complex genomes and the results used to develop RDA-derived markers for an array of applications.

Key words

Cryptococcus neoformans, *Cryptococcus gattii*, Representational Difference Analysis, polymorphism, genome.

Introduction

The two species *Cryptococcus neoformans* and *Cryptococcus gattii* are basidiomycete yeasts that infect both immunocompetent and immunocompromised individuals. *C. neoformans* includes two varieties *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) and a hybrid between the two varieties (serotype AD). *C. gattii* comprises the serotypes B and C [1]. The two species differ in their phenotypic characteristics, virulence profile, epidemiology, ecology and geographic distribution. The mechanisms underlying differences in host specificity and geographical distribution remain unknown. Virulence factors include capsule production, ability to grow at human body temperature (37 °C), melanin synthesis, and production of enzymes such as urease and phospholipase [2-6].

Recent reports from temperate climate regions on an outbreak occurring in healthy humans and animals on Vancouver Island (British Columbia, Canada), changed the previous concepts on the natural distribution of this species complex. Especially *C. gattii* was previously considered to be restricted to tropical and subtropical climates [7-11].

Several molecular typing methods have been used to characterize the species complex. PCR-fingerprinting and RFLP [12], AFLP [13-14], partial sequence analyses of the intergenic spacer region (IGS) [15-16] and multilocus sequence typing (MLST) [13] have shown considerable genetic divergence among the species and varieties. *C. neoformans* varieties are estimated to have diverged from *C. gattii* ~37 million years ago [17]. Recently, the genomes of two *C. neoformans* var. *neoformans* serotype D strains (JEC21 and B-3501A) were sequenced [18], and others are in process as one *C. neoformans* var. *grubii*

strain (H99) and two *C. gattii* strains (R265 and WM276) (http://cneo.genetics.duke.edu/; http://www.broad.mit.edu/; http://www.bcgsc.ca).

Representational difference analysis (RDA) is a PCR based subtractive enrichment procedure for isolating the differences between two nearly identical genomes [19]. This methodology was subsequently modified for cloning of differentially expressed RNA populations [20] and was applied to procaryotic and eucaryotic microorganisms [21-25]. Bailão *et al*. [23] analyzed two cDNA populations of *Paracoccidioides brasiliensis* in host interactions conditions and provide the first view of the transcriptional response of a pathogenic fungus using RDA.

Recently, the feasibility of RDA to examine the transcriptome of *C. neoformans* as a function of temperature was demonstrated. Genes were identified whose products are related to important biologic processes as the maintenance of the cell wall, oxidative metabolism and stress response [21].

The purpose of this study was to isolate DNA sequences specific to *C. gattii* to serve as a platform to design specific probes for species identification, and to provide genetic markers to better understand the epidemiology of cryptococcosis. This is the first study with RDA identifying genomic differences in human pathogenic yeasts.

Materials and Methods

Organisms and genomic DNA isolation. Two clinical strains, *C. neoformans* var. *grubii* HC6 (serotype A, molecular type VNI, mating type α) and *C. gattii* AL33 (serotype B, molecular type VGIII, mating type α), were used for the RDA

experiments [26]. The strains were kindly provided by the Hospital das Clínicas de Porto Alegre and Hospital Santa Casa de Misericórdia, in Porto Alegre, RS, Brazil. In addition the *C. neoformans* var. *neoformans* strain A45 (serotype D, molecular type VNIV, mating type α) was used, which was isolated from a eucalyptus tree [27]. For DNA extraction, the yeast was grown for 48 h at 30 °C in Sabouraud dextrose agar (Difco, Detroit, MI). For cloning *Escherichia coli* XL1 Blue competent cells and the pUC18 cloning vector containing ampicillin-resistance determinant [28] were used in this study. Total DNA was extracted from tester and driver cultures by using a modified protocol previously described by Zhang *et al*. [29].

Representational difference analysis. A modified RDA protocol was developed based on the method described by Pastorian *et al*. [30]. The oligonucleotides used for RDA were: JBam12 (5' GATCCGTTCATG 3'); JBam24 (5' ACCGACGTCGACTATCCATGAACG 3'); NBam12 (5' GATCCTCCCTCG 3'); NBam24 (5' AGGCAACTGTGCTATCCGAGGGAG 3'); RBam12 (5' GATCCTCGGTGA 3'); and RBam24 (5' AGCACTCTCCAGCCTCTCTCACCGAG 3'). Total DNA of the *C. gattii* strain AL33 (1 μg) was used as tester and total DNA of *C. neoformans* var. *grubii* strain HC6 (1 μg) was used as driver. Both DNAs were digested to completion with the restriction endonuclease Sau3AI (Amersham Pharmacia Biotech, Piscataway, NY). The resulting products were purified using the GFX kit (Amersham Pharmacia Biotech).

Briefly, for the generation of the first differential product (DP1) the RBam 12/24 adapter was ligated only to the tester, and driver and tester DNAs were

mixed at a 10:1 ratio, hybridized for 24 h at 67 °C, and amplified by PCR with RBam24 primer (7 cycles of 45 s at 95 °C and 3 min at 72 °C). The PCR product was submitted to a new round of amplification (20 cycles) aiming to remove unwanted single-stranded DNA. A final PCR amplification step produced DP1. To generate the second and third differential products (DP2 and DP3), RBam adapters was replaced by the NBam and JBam adapters and the driver to tester ratio was raised to 100:1 and 1000:1 respectively.

Analysis of RDA clones. DNA fragments from third round RDA were filled in with Klenow fragment polymerase and phosphorylated with T4 polynucleotide kinase (Amersham Pharmacia Biotech). Upon heat inactivation, the samples were purified using the GFX kit and ligated to the SmaI digested and dephosphorylated pUC18 vector. *E. coli* XL1 Blue competent cells were transformed with the ligation products. Recombinants were selected by blue/white screening and ampicillin resistance. Plasmid DNA was prepared from selected clones and sequenced with the Dyenamic ET Dye Terminator cycle sequencing kit for MegaBace DNA analysis systems (Amersham Pharmacia Biotech), using the universal primers (forward and reverse primers) for the pUC18 vector. The PHRED program was utilized to base-call nucleotide sequences of the DNA clones [31]. The resulting sequences were compared to the GenBank database using the BLASTN program [32].

Southern blot analysis and PCR assay. Total DNA (3 μg) from *C. neoformans* and *C. gattii* was EcoRI-digested and probed with selected RDA DNA fragments labelled with [α-^{32}P]-dCTP (Rediprime™ II DNA Labelling System, Amersham

Pharmacia Biotech) after agarose gel electrophoresis and capillary transfer to Hybond N+ Nylon membrane (Amersham Pharmacia Biotech) following standard procedures [28]. Hybridization conditions were as recommended by the manufacturer at 60 °C.

For PCR amplification oligonucleotide primers were designed to the insulin degrading enzyme gene (*IDE*) based on the corresponding sequence of the *C. neoformans* var. *neoformans* strain JEC21 (GenBank accession nº **XM_568105**). The primers were tested on genomic DNA isolated from different clinical and environmental strains: seven *C. gattii* serotype B isolates, seven *C. neoformans* var. *grubii* serotype A isolates and three *C. neoformans* var. *neoformans* serotype D isolates. To 25 μL final reaction volume were added: 20 to 30 ng of total DNA, 1 U *Taq* polymerase, 20 pmol each primer, 2.5 mM dNTPs (dATP, dCTP, dGTP, dTTP) and 2 mM $MgCl_2$. The following oligonucleotides were used: IDE forward (5' CCAAGGCGGACAAGGCTGCGG 3') and IDE reverse (5'-GTAGAGGTGATCCATGTCGGG 3'). PCR cycles were programmed as follows: 5 min at 95 °C followed by 30 cycles (95 °C for 45 s, 67 °C for 40 s, 72 °C for 1 min). The PCR products were checked on a 1% agarose gel, purified with Yeast tRNA (Invitrogen, Carlsbad, CA), sequenced and analyzed using the softwares Staden [33], ClustalX [34] and GeneDoc [35].

Results

Isolation of DNA sequences from *C. gattii* by representational difference analysis. The first round of subtractive hybridization and kinetic enrichment decreased the complexity of the *C. gattii* genome to a smear from approximately 1,000 bp to 200 bp. After three rounds of RDA, agarose gel

electrophoresis of the amplified DNA fragments revealed one major band and a decrease in the relative intensity of the minor bands that were present in the second round (Figure 1). Although, in the first round a smear of DNA products was detected, a different amplified DNA fragment pattern was evident even after two rounds of RDA using different driver to tester ratios.

The resulting RDA fragment population was cloned into the pUC18 vector and approximately 200 randomly picked clones were sequenced and Blast searched in GenBank. One hundred and sixty one clones showed homology to known 19 different sequences, while the others had no significant matches as detected by the BLASTN program. Sequences with E_{values} $<10^{-5}$ were considered, as outlined in Table 1. As expected, the clones showed significant similarity to the genome sequence of *C. neoformans* var. *neoformans* JEC21 strain and with the other *Cryptococcus* genomes (B-3501, H99, R265, WM276). The genes shown in Table 1 encode several putative proteins classified as unknown proteins, enzymes involved in metabolic pathways, factors related to cell division cycle, response to stresses and transcription regulator factors.

Copy number and polymorphism of selected sequences. Southern hybridization experiments were performed to examine whether some of the sequences identified by RDA were unique to the *C. gattii* tester strain. One out of five sequences tested hybridized only with total DNA of the *C. gattii* tester strain (Figure 2A). BLASTN searches of the GenBank database revealed a similarity between this sequence and a region of the conserved hypothetical protein CNL05140, described in the genome of *C. neoformans* var. *neoformans*

strain JEC21. The cloned DNA fragment of 987 bp in length of this ORF strongly hybridized to the genomic DNA of the *C. gattii* strain AL33 but not to the *C. neoformans* var. *grubii* strain HC6. Another sequence that encodes a putative endoplasmic reticulum protein (CND02670) also revealed differences in the hybridization patterns of digested genomic DNAs of *C. gattii* and *C. neoformans* var. *grubii* (Figure 2B) with the presence of two bands in the former (approximately 5.0 and 1.8 Kb) and only one band in the latter (minor than 5.0 Kb).

The comparison between the hybridization patterns of the sequences from the putative insulin degrading enzyme (IDE, CNL05710) and chitin synthase (CNC00050) against three isolates of the *Cryptococcus* species complex revealed polymorphisms between the two species. The product of locus CNL05710 (IDE) has a metallopeptidase activity and it is involved in the maturation of peptide pheromone, proteolysis and peptidolysis reactions. The product of locus CNC00050, a putative chitin synthase, is described as involved in cell budding. However, the hybridization patterns of the sequence that encodes the putative proteasome subunit alpha type 5, (CNH01360) was similar for strains HC6, AL33 and A45; therefore, this sequence is not specific for *C. gattii* and has no applicability as PCR-based marker (Figure 3).

To confirm the polymorphism found for the putative *IDE* gene (Southern blotting, Figure 3), the amplicons from *C. neoformans* and *C. gattii* were purified and sequenced and the consensus sequence of each amplicon was generated by the Staden program. Figure 4 depicts a schematic representation of the localization of the *IDE* identified RDA product, the PCR amplicon and the sequence of locus CNL05710 on the chromosome 12 (GenBank accession nº

AE017352). The sequence alignment showed conserved regions in the nucleotide sequences of the seventeen amplicons, with 86% identity between serotype B and D and no more than 36% identity between serotypes A and B or D.

Discussion

RDA rendered the selection of polymorphic sequences and this was possible even when the two species under investigation were highly similar in their genome complexity [36]. The differences found between the two genomes analyzed, *C. gattii* and *C. neoformans* var. *grubii* can be easily converted into PCR-based markers to allow for the discrimination between taxa, based on the presence of specific amplicons.

We initially focused our attention on the differences between *C. gattii* and *C. neoformans* var. *grubii* since these are the most common pathogenic *Cryptococcus* species. However, the polymorphism verified in the first results of the hybridization analysis indicated the necessity to investigate a strain of *C. neoformans* var. *neoformans* in Southern experiments, for which we choose an environmental strain A45. The polymorphism identified between *C. gattii* and the *C. neoformans* varieties for the sequences from a putative *IDE*, chitin synthase and the endoplasmic reticulum protein, highlight the variability that exist into the *Cryptococcus* species complex [1]. The observation that there are polymorphisms even between the *C. neoformans* isolates from *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* is interesting because it demonstrates possible genetic variability in other sequences yet not studied in this species. Moreover, the signal intensities of *C. neoformans* samples in

Southern experiments were relatively weak when compared with those of *C. gattii* samples, suggesting that the probed genes are less abundant in the *C. neoformans* genome or that shows a poorer sequence homology with the hybridization probe.

Five sequences selected by RDA were chosen for further studies. These sequences were classified either as of unknown function or hypothetic conserved and their function is not known. One of the sequences is the putative *IDE* gene. The Ste23p and Axl1p proteins from *Saccharomyces cerevisiae* have significant sequence homology to human IDE and genetic and mutational studies indicate that these proteins are required for the proteolytic maturation of the yeast *a*-factor mating pheromone. Axlp1 is also required for maintenance of the axial budding pattern characteristic of haploid yeast [37-38]. Kim *et al*. [39] have demonstrated that the Axlp1/Ste23p-dependent step in *a*-factor production can be supported by IDE. The *IDE* gene is highly conserved during evolution from *E. coli* to human; exons and introns retained identity in different organisms [40]. The sequence isolated in this work with identity to *IDE* revealed polymorphism among *C. neoformans* and *C. gattii* as shown by Southern blotting. The polymorphism was confirmed by sequencing of the PCR products from part of putative *IDE* gene of serotypes A, B and D isolates and demonstrated that the isolates from serotype A are clearly different at the nucleotide sequence level from other serotypes tested. However, only examples of each serotype have been sequenced and this is stating simply the polymorphisms found between those serotypes. These data still are not enough to state if the polymorphism found indicates a variation that may be segregating in a population or a fixed difference between serotypes/varieties. The

sequencing of several strains of the same serotype, including serotypes A, B, C, D and AD, is being carried out (Ngamskulrungroj *et al.*, unpublished results). *C. neoformans* has eight putative chitin synthases, and strains with any one deleted gene of chitin synthase are viable at 30 °C. *C. neoformans* has three genes that encode putative regulator proteins, which are homologues of *S. cerevisiae* Skt5p. One of the chitin synthases, chitin synthase 3 (Chs3), and one of the regulators (Csr2) are important for growth [41]. The sequence of putative chitin synthase gene isolated in this work by RDA revealed polymorphisms among the three isolates used in the Southern blotting experiments. Other studies about this gene function and relevance may be useful for the *C. neoformans* species characterization, as according with the observations of Walton *et al.* [42], the molecular and genetic components of virulence pathways, as the Chs3, are potentially novel DNA markers or targets for drug development against *C. neoformans*.

One limitation of all subtractive hybridization methods is the presence of a certain proportion of false positives [43-44]. To avoid this, a recent work described a method denominated hybridization-monitored genome differential analysis (HMDA). This technique incorporates a monitor system into a PCR-based solid subtraction hybridization that tracks the entire hybridization process using PCR analysis of the conserved sequence in the tester sample after each round of subtraction [45]. However, this method is not widely tested yet and our work demonstrated that RDA, when properly performed, is successfully applied to the identification of differences between two complex genomes and not only to the low-complexity genomes of prokaryotic organisms and the results can be used to develop RDA-derived markers for other applications.

The mechanisms underlying differences in host predilection and geographical distribution of the *Cryptococcus* species complex remain unknown. In many studies based on conserved sequences from the *Cryptococcus* species complex no significant differences were seen in the genetic structure of different strains of *C. neoformans* var. *neoformans* isolated from different geographic locations. Other works showed that there is a geographical differentiation among *C. gattii* isolates [14,16] and even between serotype A strains [46]. The use of other sequences may be useful to help to study the genetic structure and geographic distribution of clinical and environmental isolates. Strain-specific DNA-based diagnostic tools for rapid strain detection and identification could be developed, which may use novel sequences identified by RDA to determine strain variation and provide insights into evolutionary processes.

Finally, this is the first report of a genomic RDA application for pathogenic yeasts and may lead to the study of new genes in *C. gattii*. Therefore, future work will be needed to investigate some of these fragments, which may help to begin the elucidation of the basic biology of the strain differences and the understanding of the microbial diversity.

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References

1. Kwon-Chung KJ, Boekhout T, Fell JW, Diaz M. Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacillisporus* (Basidiomycota, Hymenomycetes,Tremenomycetidae). *Taxon* 2002; **51:** 804–806.
2. Okabayashi K, Kano R, Watanabe S, Hasegawal A. Expression of capsule-associated genes of *Cryptococcus neoformans*. *Mycopathologia* 2005; **160:** 1–7.
3. Latouche GN, Sorrell TC, Meyer W. Isolation and characterization of the phospholipase B gene of *Cryptococcus neoformans* var. *gattii. FEMS Yeast Res* 2002; **2:** 551-561.
4. Cox GM, McDade HC, Chen SC, *et al*. Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. *Mol Microbiol* 2001; **39:** 166–175.
5. Cox GM, Mukherjee J, Cole GT, Casadevall A, Perfect JR. Urease as a virulence factor in experimental cryptococcosis. *Infect Immun* 2000; **68:** 443–448.
6. Casadevall A, Perfect JR. *Cryptococcus neoformans*. Washington, DC: The American Society for Microbiology, 1998.
7. Kidd SE, Bach PJ, Hingston AO, *et al*. *Cryptococcus gattii* dispersal mechanism, British Columbia, Canada. *Emerg Infect Dis* 2007; **13:** 51-57.
8. 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.

9. MacDougall L, Fyfe M. Emergence of *Cryptococcus gattii* in a novel environment provides clues to its incubation period. *J Clin Microbiol* 2006; **44:** 1851-1852.

10. Hoang LM, Maguire JA, Doyle P, Fyfe M, Roscoe DL. *Cryptococcus neoformans* infections at Vancouver Hospital and Health Sciences Centre (1997–2002): Epidemiology, microbiology and histopathology. *J Med Microbiol* 2004; **53:** 935–940.

11. Sorrell TC. *Cryptococcus neoformans* variety *gattii*. *Med Mycol* 2001; **39:** 155–168.

12. Meyer W, Castañeda A, Jackson S, *et al*. Molecular typing of iberoamerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis* 2003; **9:** 189–195

13. Litvintseva AP, Thakur R, Vilgalys R, Mitchell TG. Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (serotype A), including a unique population in Botswana. *Genetics* 2006; **172:** 2223-2238.

14. Boekhout T, Theelen B, Diaz M, *et al*. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* 2001; **147:** 891-907.

15. Diaz MR, Boekhout T, Kiesling T, Fell JW. Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast *Cryptococcus neoformans*. *FEMS Yeast Res* 2005; **5:** 1129-1140.

16. Diaz MR, Boekhout T, Theelen B, Fell JW. Molecular sequence analyses of the intergenic spacer (IGS) associated with rDNA of the two varieties

of the pathogenic yeast, *Cryptococcus neoformans. System Appl Microbiol* 2000; **23:** 535-545.

17. Xu J, Vilgalys R, Mitchell TG. Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. *Mol Ecol* 2000; **9:** 1471-1481.

18. Loftus BJ, Fung E, Roncaglia P, *et al*. The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. *Science* 2005; **307:** 1321–1324.

19. Lisitsyn N, Lisitsyn N, Wigler M. Cloning the differences between two complex genomes. *Science* 1993; **259:** 946-951.

20. Hubank M, Schatz DG. Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res* 1994; **22:** 5640-5648.

21. Rosa e Silva LK, Staats CC, Goulart LS, *et al.* Identification of novel temperature-regulated genes in the human pathogen *Cryptococcus neoformans* using representational difference analysis. *Res Microbiol* 2008; doi:10.1016/j.resmic.2007.12.006.

22. Alzate JF, Ramírez-Pineda JR, González VM, *et al. Leishmania (Viannia) panamensis*: Cloning of the histone H1 genes by representational difference analysis. *Exp Parasitol* 2006; **112:** 126-129.

23. Bailão AM, Schrank A, Borges CL, *et al*. Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: Representational difference analysis identifies candidate genes associated with fungal pathogenesis. *Microbes Infect* 2006; **8:** 2686-2697.

24. Dutra V, Nakasato L, Broetto L, *et al*. Application of representational difference analysis to identify sequence tags expressed by *Metharizium anisopliae* during the infection process of the tick *Boophilus microplus* cuticle. *Res Microbiol* 2004; **155:** 245-251.

25. Bowler LD, Hubank M, Spratt BG. Representational difference analysis of cDNA for the detection of differential gene expression in bacteria: development using a model of iron-regulated gene expression in *Neisseria meningitidis*. *Microbiology* 1999; **145:** 3529-3537.

26. Casali AK, Goulart L, Silva LKR, *et al*. Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *FEMS Yeast Res* 2003; **3:** 405-415.

27. Medeiros Ribeiro A, Silva LK, Silveira Schrank I, *et al*. Isolation of *Cryptococcus neoformans* var. *neoformans* serotype D from Eucalypts in South Brazil. *Med Mycol* 2006; **44:** 707-713.

28. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: a Laboratory Manual, 2nd eds. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 2001.

29. Zhang D, Yang Y, Castlebury LA, Cerniglia CE. A method for the large scale isolation of high transformation efficiency fungal genomic DNA. *FEMS Microbiol Lett* 1996; **145:** 261-265.

30. Pastorian K, Hawell L, Byus CV. Optimization of cDNA representational difference analysis for the identification of differentially expressed mRNAs. *Anal Biochem* 2000; **283:** 89-98.

31. Ewing B, Hillier LM, Wendl C, Green P. Base-calling of automated sequencer traces using PHRED. I. Accuracy assessment. *Genome Res* 1998; **8:** 175–185.

32. Altschul SF, Madden TL, Schaffer AA, *et al*. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 1997; **25:** 3389-3402.

33. Staden R, Beal KF, Bonfield JK. The Staden package. *Methods Mol Biol* 2000; **132:** 115-130.

34. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; **25:** 4876-4882.

35. Nicholas KB, Nicholas Jr. HB, Deerfiled DW. GeneDoc: a tool for editing and annotating multiple sequence alignments. *EMBnet News* 1997; **4:** 14.

36. Del Rio ML, Navas-Mendez J, Gutierrez-Martin CB, Rodriguez-Barbosa JI & Rodriguez-Ferri EF. Identification of *sul*I allele of dihydropteroate synthase by representational difference analysis in *Haemophilus parasuis* serovar 2. *Lett Appl Microbiol* 2005; **40:** 436-442.

37. Adames N, Blundell K, Ashby MN, Boone C. Role of yeast insulin-degrading enzyme homologs in propheromone processing and bud site selection. *Science* 1995; **270:** 464–467.

38. Fujita A, Oka C, Arikawa Y, *et al*. A yeast gene necessary for bud-site selection encodes a protein similar to insulin-degrading enzymes. *Nature* 1994; **372:** 567–570.

39. Kim S, Lapham AN, Freedman CGK, Reed TL, Schmidt WK. Yeast as a tractable genetic system for functional studies of the insulin-degrading enzyme. *J Biol Chem* 2005; **280:** 27481-27490.

40. Qiu WQ, Folstein MF. Insulin, insulin-degrading enzyme and amyloid-peptide in Alzheimer's disease: review and hypothesis. *Neurobiol Aging* 2006; **27:** 190-198.

41. Banks IR, Specht CA, Donlin MJ, *et al*. A chitin synthase and its regulator protein are critical for chitosan production and growth of the fungal pathogen *Cryptococcus neoformans*. *Eucaryot Cell* 2005; **4:** 1902-1912.

42. Walton FJ, Idnurm A, Heitman J. Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans*. *Mol Microbiol* 2005; **57:** 1381-1396.

43. Agron PG, Macht M, Radnedge L, *et al*. Use of subtractive hybridization for comprehensive surveys of procaryotic genome differences. *FEMS Microbiol Lett* 2002; **211:** 175-182.

44. Sagerström CG, Sun BI, Sive HL. Subtractive cloning: Past, present and future. *Annu Rev Biochem* 1997, **66:** 751-783.

45. Cao Y, He Z, Wang Z, *et al*. Hybridization monitor: A method for identifying differences between complex genomes. *J Microbiol Meth* 2006; **64:** 305-315.

46. Litvintseva AP, Marra RE, Nielsen K, *et al*. Evidence of sexual recombination among *Cryptococcus neoformans* serotype A isolates in sub-Saharan Africa. *Eukaryot Cell* 2003; **2:** 1162-1168.

Figure captions

Figure 1. Agarose gel electrophoresis of total digested DNA and subtractive differential DNA pools derived from *C. gattii* (strain AL33). **(A)** Lane 1, total DNA of *C. gattii* (strain AL33), lane 2, total DNA of *C. gattii* (strain AL33) digested with Sau3AI, lane 3, total DNA of *C. neoformans* var. *grubii* (strain HC6), lane 4, total DNA of *C. neoformans* var. *grubii* (strain HC6) digested with Sau3AI. **(B)** Differential fragments of DNA of *C. gattii* (strain AL33) from first (lane 1), second (lane 2) and third (lane 1) rounds of RDA. The sizes of DNA fragments are indicated on the left side (M).

Figure 2. Southern blot analysis of RDA selected sequences using *C. gattii* (strain AL33) as *tester* and *C. neoformans* var. *grubii* (strain HC6) as *driver*. Total DNA was digested with EcoRI and probed with radiolabelled fragments from the third round of RDA: **(A)** probed with the conserved hypothetical protein (CNL05140) and, **(B)** probed with the putative endoplasmic reticulum protein (CND02670). Molecular size markers are indicated in kilobase pairs.

Figure 3. Southern blot analysis of RDA clones against total DNA from strains A45 (lane 1, *C. neoformans* var. *neoformans*), HC6 (lane 2, *C. neoformans* var. *grubii*) and AL33 (lane 3, *C. gattii*). Total DNA was digested with EcoRI and probed with radiolabelled fragments from third round of RDA. In **(A)** chitin synthase, putative (CNC00050), **(B)** insulin degrading enzyme, putative (CNL05710) and **(C)** proteasome subunit alpha type 5, putative (CNH01360). Molecular weight markers are indicated in kilobase pairs on the right.

Figure 4. PCR analysis of the product of RDA corresponding to the insulin degrading enzyme (*IDE*) gene. **(A)** Schematic representation of the deduced location of the PCR amplicon with designed primers (arrows), the product of RDA and the region corresponding to locus *IDE* (CNL05710) on the chromosome 12 (GenBank accession nº **AE017352**). **(B)** Alignment generated with Clustal X and GeneDoc programs of *IDE* amplicons (~860 bp) of the strains of three serotypes A, D and B (ser_A, ser_D and ser_B, respectively) with part of the corresponding sequence of *IDE* from *C. neoformans* var. *neoformans* JEC21 (IDE_CNL05710). The regions of primers used in the PCR are underlined.

Table 1. Summary of the BLASTN search of the sequenced clones isolated by RDA

Description[a]	Accession N°[b]	Clone redundancy[c]	BlastN (E value)[d]	Chromosome N°[e]
Beta-1,3-glucan biosynthesis-related protein, putative (CNK02770)	XM_567874	1	2e-32	11
Chitin synthase, putative (CNC00050)	XM_569357	12	2e-17	3
Endoplasmic reticulum protein, putative (CND02670)	XM_570239	18	8e-116	4
Proteasome subunit alpha type 5, putative (CNH01360)	XM_572324	2	2e-79	8
Mitochondrion protein, putative (CNA03910)	XM_566735	4	2e-76	1
Transcription factor, putative (CND00200)	XM_570377	2	2e-22	4
Gim complex component GIM3 (CNL05150)	XM_568055	9	2e-17	12
Conserved hypothetical protein (CNL05140)	XM_568054	39	1e-40	12
Conserved hypothetical protein (CNA05040)	XM_566792	3	2e-29	1
Hypothetical protein (CNB00810)	XM_569089	2	2e-122	2
Hypothetical protein (CND03970)	XM_570631	2	1e-43	4

Conserved hypothetical protein (CNG04150)	XM_572169	1	1e-107	7
Carbonic anhydrase 2 (CAN2)*	DQ115790	1	5e-11	Nd
PAK1 kinase gene*	AF391150	2	4e-13	Nd
Mating type alpha locus**	DQ096809	1	6e-147	Nd
Nuclear membrane protein, putative (CNE02760)	XM_570985	1	2e-45	5
Cytoplasm protein, putative (CNC05530)	XM_569757	3	3e-74	3
Insulin degrading enzyme, putative (CNL05710)	XM_568105	4	4e-101	12
Filobasidiella neoformans var. neoformans 5S ribosomal RNA, 17S ribosomal RNA genes, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 25S ribosomal RNA gene	AF356652	54	9e-162	

[a] Description of homologous gene product in *C. neoformans* var. *neoformans* strain jec21, *C. neoformans* var. *grubii* strain H99 (*) or *C. gattii* strain R265 (**).

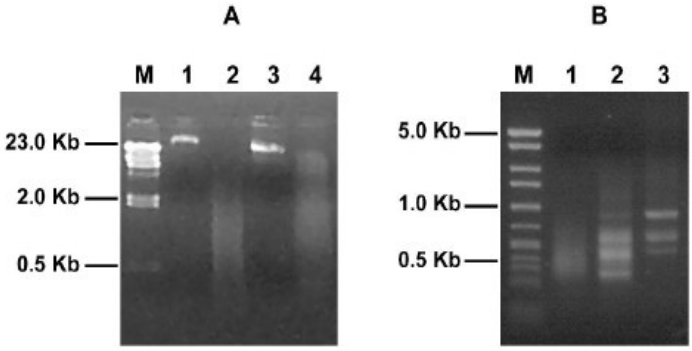
[b] Accession number of homologous gene product in the GenBank database.

[c] The number of times that the corresponding clone appeared in the experiments.

[d] *E*-value (expected value) is analyzed by BLASTN.

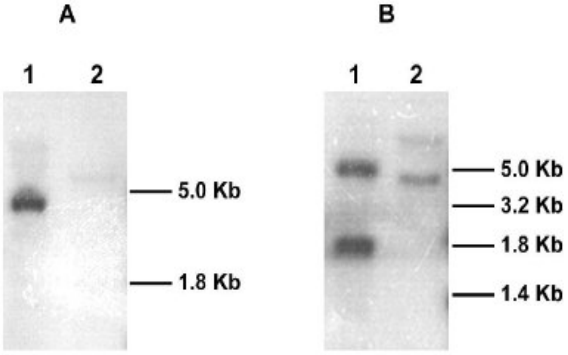
[e] Number of corresponding chromosome where the sequences are localized from the genome sequenced *C. neoformans* var. *neoformans* strain JEC21.

Nd – not assigned



Agarose gel electrophoresis of total digested DNA and subtractive differential DNA pools derived from C. gattii (strain AL33). (A) Lane 1, total DNA of C. gattii (strain AL33), lane 2, total DNA of C. gattii (strain AL33) digested with Sau3AI, lane 3, total DNA of C. neoformans var. grubii (strain HC6), lane 4, total DNA of C. neoformans var. grubii (strain HC6) digested with Sau3AI. (B) Differential fragments of DNA of C. gattii (strain AL33) from first (lane 1), second (lane 2) and third (lane 1) rounds of RDA. The sizes of DNA fragments are indicated on the left side (M).

113x45mm (500 x 500 DPI)



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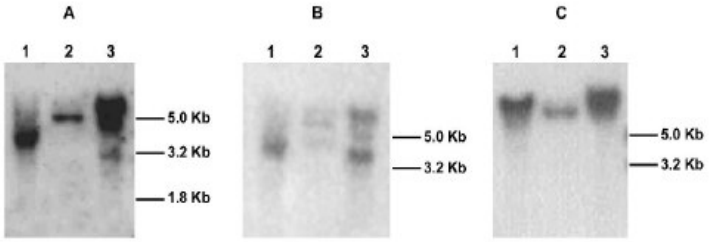
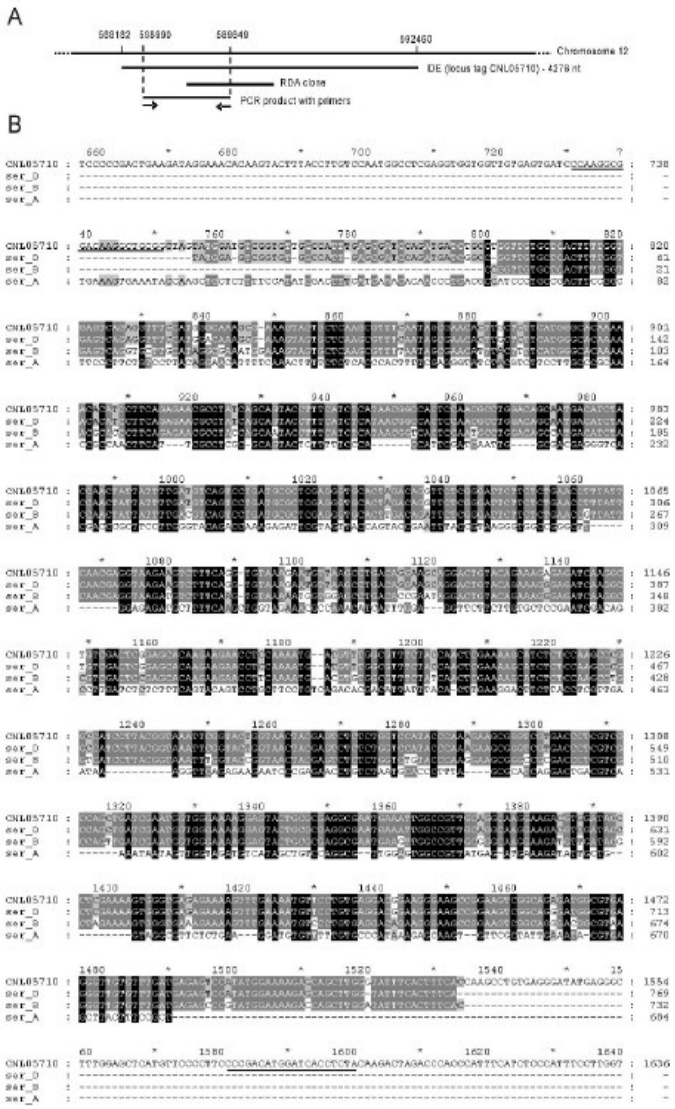


Southern blot analysis of RDA clones against total DNA from strains A45 (lane 1, C. neoformans var. neoformans), HC6 (lane 2, C. neoformans var. grubii) and AL33 (lane 3, C. gattii). Total DNA was digested with EcoRI and probed with radiolabelled fragments from third round of RDA. In (A) chitin synthase, putative (CNC00050), (B) insulin degrading enzyme, putative (CNL05710) and (C) proteasome subunit alpha type 5, putative (CNH01360). Molecular weight markers are indicated in kilobase pairs on the right.

167x45mm (500 x 500 DPI)



PCR analysis of the product of RDA corresponding to the insulin degrading enzyme (IDE) gene. (A) Schematic representation of the deduced location of the PCR amplicon with designed primers (arrows), the product of RDA and the region corresponding to locus IDE (CNL05710) on the chromosome 12 (GenBank accession nº AE017352). (B) Alignment generated with Clustal X and GeneDoc programs of IDE amplicons (~860 bp) of the strains of three serotypes A, D and B (ser_A, ser_D and ser_B, respectively) with part of the corresponding sequence of IDE from C. neoformans var. neoformans JEC21 (IDE_CNL05710). The regions of primers used in the PCR are underlined.
167x257mm (500 x 500 DPI)

Fungal Genetics and Biology

4.1.2. Genetic diversity of the *Cryptococcus* species complex: Does *Cryptococcus gattii* deserve to have varieties?

Popchai Ngamskulrungroj, Josiane Faganello, Kin Ming Tsui, Ana Lusia Leal,

Marilene Henning Vainstein and Wieland Meyer

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Abstract: The Cryptococcus species complex that causes central meningoencephalitis is composed of the basidiomycetous yeasts Cryptococcus neoformans (var. grubii and var. neoformans) and Cryptococcus gattii. Three haploid molecular types, VNI, VNII and VNIV, and 4 haploid molecular types, VGI, VGII, VGIII and VGIV have been recognized in C. neoformans and C. gattii repectively. DNA sequence data of 4 loci obtained from 73 cryptococcal strains collected from 6 continents, recognized each of the molecular type associated clades with an exception of 3 VNII strains and 3 VGIV strains, which clustered with VNI and VGIII respectively. Based on PLB1 sequences, we found the Botswana VNB population was also presented in other parts of the world and clustered with the molecular type VNI clade. The molecular type VGI, VGIII and VGIV clades (CGa) formed a sister relationship with the VGII clade (CGb) with a high support from both parsimony and likelihood analysis and possibly deserve a variety level.

Genetic diversity of the *Cryptococcus species* complex: Does *Cryptococcus gattii* deserve to have varieties?

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Abstract:

The *Cryptococcus species* complex that causes central meningoencephalitis is composed of the basidiomycetous yeasts *Cryptococcus neoformans* (var. *grubii* and var. *neoformans*) and *Cryptococcus gattii*. Three haploid molecular types, VNI, VNII and VNIV, and 4 haploid molecular types, VGI, VGII, VGIII and VGIV have been recognized in *C. neoformans* and *C. gattii* repectively. DNA sequence data of 4 loci obtained from 73 cryptococcal strains collected from 6 continents, recognized each of the molecular type associated clades with an exception of 3 VNII strains and 3 VGIV strains, which clustered with VNI and VGIII respectively. Based on *PLB1* sequences, we found the Botswana VNB population was also presented in other parts of the world and clustered with the molecular type VNI clade. The molecular type VGI, VGIII and VGIV clades (CGa) formed a sister relationship with the VGII clade (CGb) with a high support from both parsimony and likelihood analysis and possibly deserve a variety level.

Key words: Molecular type, *Cryptococcus*, phylogenetic analysis, speciation, population study

47 **Introduction:**

48

49 The *Cryptococcus species* complex that causes cryptococcal meningoencephalitis
50 is composed of two basidiomycetous encapsulated yeasts species: *Cryptococcus*
51 *neoformans*, an opportunistic pathogen, and *Cryptococcus gattii,* a primary pathogen,
52 which are considered as the most common fungal pathogens causing infection of the
53 central nervous system (Casadevall and Perfect, 1998). Two varieties, *C. neoformans* var.
54 *grubii* (serotype A), found worldwide, and *C. neoformans* var. *neoformans* (serotype D),
55 mainly present in Europe and South America, have been recognized within the species *C.*
56 *neoformans*. *C. gattii* (Kwon-Chung et al., 2002), previously known as *C. neoformans*
57 *var. gattii* (serotype B and C), was thought to be restrict to tropical and subtropical
58 climate zones (Kwon-Chung and Bennett, 1984; Sorrell, 2001) until a recent outbreak of
59 cryptococcosis occurred on Vancouver Island, Canada, which has expanded the
60 ecological niche of this yeast to a temperate region (Kidd et al., 2004).

61 In recent years a large array of molecular studies, including PCR-fingerprinting
62 (Meyer et al., 1999), AFLP analysis (Boekhout et al., 2001), RFLP analysis of the
63 orotidine monophosphate pyrophosphorylase (*URA5*) (Meyer et al., 2003) and
64 phospholipase (*PLB1*) (Latouche et al., 2003), have grouped all globally obtained strains
65 into eight distinct major molecular types. The comparable molecular types are as follows:
66 VNI (=AFLP1) and VNII (=AFLP1A) (*C. neoformans* var. *grubii*); VNIV (=AFLP2) (*C.*
67 *neoformans* var. *neoformans*); VNIII (=AFLP3) (hybrid serotype or serotype AD); and
68 VGI (=AFLP4), VGII (=AFLP6), VGIII (=AFLP5) and VGIV (=AFLP7), all
69 corresponding to serotypes B and C (*C. gattii).* In addition, also hybrid strains within *C.*

gattii were reported but no specific molecular type(s) has been designated yet (Boekhout et al., 2001). Similar sub-groupings, representing this level of genetic heterogeneity have been found in subsequent sequences analysis studies (Butler and Poulter, 2005; Diaz et al., 2005; Litvintseva et al., 2005; Xu et al., 2000). The degree of variation between the eight molecular types indicated that the varieties and species are genetically distinct and that there is an ongoing evolutionary divergence between these eight molecular types (Diaz et al., 2005).

The present study aimed to investigate the phylogenetic relationship of species and varieties in order to test the monophyly of each molecular type among members of *Cryptococcus species* complex based on the sequence variation patterns across house keeping, enzyme encoding and virulence genes, namely: actin gene (*ACT1*) (Cox et al., 1995), orotate-phosphoribosyl transferase encoding gene (*URA5*) (Franzot et al., 1998) and phospholipase B encoding gene (*PLB1*) (Cox et al., 2001). In addition, an Insulin degrading enzyme encoding gene (*IDE*), a 110-kDa neutral metalloendopeptidase, was also chosen since it has been known to be conserved across wide range of organisms (Chesneau and Rosner, 2000; Duckworth et al., 1998; Garcia et al., 1989) and could be used as a tool for studying phylogenetic relationship between species (Authier et al., 1995).

Materials and Methods:

Studied strains: Seventy four strains composed of at least ten strains of each haploid molecular type of the *Cryptococcus species* complex from different parts of the

93 world and *Cryptococcus albidus* were retrieved form the Molecular Mycology Research
94 Laboratory culture collection at Westmead Hospital (Table 1). The hybrid molecular type
95 was excluded from the study due to ambiguity of phylogenetic resolution in terms of
96 variation in numbers of copies of each locus which deserves to be studied separately
97 (Litvintseva et al., 2007; Xu and Mitchell, 2003). The strains were maintained on
98 Saboraud Dextose Agar (2% glucose, 2% peptone and 2% agar) 72 hours before DNA
99 extraction.

100 **DNA extraction, molecular type verification, amplification and sequencing:**
101 DNA extractions were performed by liquid nitrogen grinding method as previously
102 described (Meyer et al., 2003). Genomic DNA of *Filobasidiella depauperata* was kindly
103 provided by Ferry Hagen, the Centraalbureau voor Schimmelcultures, Utrecht, the
104 Netherlands. *URA5*-RFLP analysis was first performed to verify the molecular type of
105 each studied strain as previously described (Meyer et al., 2003). The *ACT1* (Cox et al.,
106 1995), *URA5* (Franzot et al., 1998), *PLB1* (Cox et al., 2001) and *IDE* (Authier et al.,
107 1995; Garcia et al., 1989) genes were amplified from each strain with the primers
108 presented in Table 2. Each polymerase chain reaction (PCR) contained 50 ng of genomic
109 DNA, 50 ng of each primers, 0.2 mM of dNTP, 3mM of $MgCl_2$ and 0.5 unit of either
110 AmpliTaq® (Applied biosystems, CA, USA), BioTaq® (Bioline, NSW, Australia) or
111 ExTaq® (Takara, Shiga, Japan) DNA polymerase with 1X of compatible buffer in a total
112 volume of 50 µl. PCR conditions for the *ACT1* gene amplification were as follows: 3 min
113 of initial denaturation at 94 ºC, followed by 35 cycles of 45 seconds at 94 ºC, 45 seconds
114 at 56 ºC, 1 min at 72 ºC and lastly, 7 min of final extension at 72 ºC. Amplification
115 conditions of the other genes were similar to that of the *ACT1* gene, but had different

116 annealing temperatures and extension times as follows: *URA5* (62 °C and 2 min); *PLB1*
117 for molecular type VNI, VNII and VNIV (60 °C and 2 min); *PLB1* for molecular type
118 VGI, VGII, VGIII and VGIV (58 °C and 2 min); and *IDE* (62 °C and 1 min). *ACT1* of *F.*
119 *depauperata* was amplified directly using the primers CNACT1 and CNACT1R primers
120 whereas the *ACT1* of *C. albidus* was amplified using the primers ACT1CAF1 and
121 ACT1CAR1 (Table 2). *URA5* of *F. depauperata* was amplified using the primers
122 URA5DF and SJ101 (Table 2). Due to a problem of non-specific bands, several *URA5*
123 amplicons of *F. depauperata* were cloned using the pGEM®-T Easy Vector System I
124 according to the manufacturer's protocol (Promega®, NSW, Australia). PCR amplicons
125 and the *URA5* plasmid were purified and sent for sequencing via the ABI Big dye
126 Terminator method at Macrogen Inc. (Korea) using the amplification and additional
127 internal primers (Table 2). Sequences were assembled using either the program
128 Sequencher version 4.6 (Gene Codes, MI, USA) or Bioedit version 7.0.5.3 (Hall, 1999).
129 Intron and exon positions were determined by aligning the obtained sequences with the
130 reference sequences of each gene as follows: *ACT1* (GenBank Accession No. **U10867**),
131 *URA5* (GenBank Accession No. **AF032436**), *PLB1* (GenBank Accession No.
132 **AF223383**), *IDE* (GenBank Accession No. **XM568105**). Sequences including and
133 excluding introns were aligned using Clustal X (Thompson et al., 1994) implemented in
134 the Bioedit program. Sequences were deposited in Genbank under accession No.
135 **EU399554** - **EU399629**, **EU408478** - **EU408653** and **EU408658** – **EU408700**.
136 Combined datasets were created by a combination of the *ACT1*, *URA5*, *PLB1* and *IDE*
137 sequences. Sequences from the closet siblings of the *Cryptococcus species* complex: *F.*

138 *depauperata and C. albidus,* were used as outgroups (Casadevall and Perfect, 1998;
139 Kidd, 2003).

140 **Mating type determination:** The mating type of each strain was determined by a
141 specific PCR as previously published (Chaturvedi et al., 2000; Fraser et al., 2003;
142 Halliday and Carter, 2003). Specific primers for each mating type are listed in Table 2.
143 The mating type **a** of the molecular types VGIII and VGIV were determined by
144 sequencing, using the primers MFa2U and MFa2L (Table 2) and performing a BlastN
145 search in the GenBank database to search for sequence homology.

146 **Phylogenetic analysis:** The phylogenetic relationships within the *Cryptococcus*
147 *species* complex were inferred by parsimony and likelihood analysis. Parsimony analysis
148 was conducted with the program PAUP* 4.0b10, using the heuristic search option
149 (Swofford, 2001). For the maximum parsimony (MP) analysis starting trees were
150 obtained via stepwise addition with 100 random sequence additions. Tree bisection-
151 reconnection was used for branch-swapping. MP phylogeny was generated for each of the
152 four loci, and the combined datasets, which included every locus of all fungal isolates.
153 Bootstrap analysis using 500 heuristic replicates was used to determine support for the
154 clades of each locus with MaxTree set to 100. Gaps were treated as missing, all
155 characters are equally weighted.

156 Bayesian phylogenetic analysis was used to determine the probability of the
157 taxonomic structure given the data at each individual locus, as well as the combined data
158 from all four loci using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). First, the
159 model of nucleotide substitution that fits best the data of each locus was determined by a
160 likelihood ratio test using the program PAUP* 4.0b10. The likelihood scores files created

161 in PAUP were then analyzed with the program ModelTest 3.7 on the ModelTest server
162 1.0 (http://darwin.uvigo.es/software/modeltest_server.html) using the Akaike information
163 criterion (AIC) (Posada and Crandall, 1998). In the analysis of the combined loci,
164 parameter estimates of each locus were unlinked, allowing independent substitution
165 models for each locus. Two analyses were performed simultaneously and used to
166 calculate the posterior probabilities, as estimated from uniform priors, for the clades of
167 each locus and the combined loci. Each analysis included four simultaneous and
168 incrementally heated Markov chains; each replicate used default heating values. Markov
169 chains were initiated from a random tree and were run for 1,000,000 generations.
170 Samples were taken every 100^{th} generation. Standard deviation of split frequencies of the
171 two runs was monitored until they converged. Last 5,000 samples of each analysis
172 containing the standard deviation less than 0.02 were used to generated concensus trees
173 by using PAUP under the 50% majority rule. The clade posterior probabilities and overall
174 topology of each replicate were compared to verify that each consensus tree converged on
175 a similar phylogeny.
176 Genetic variations of each locus were determined by using the program PAUP.
177 Total character variations and parsimony informative characters were defined both from
178 each original molecular type and from all strains of *Cryptococcus species* complex.
179
180 **Results:**
181
182 **Strains:** 10 strains from molecular type VNI, VNII, VNIV, VGI, VGIII and
183 VGIV, and 13 strains from molecular type VGII representing 6 continents were used in

this study (Table 1 and 3). Only Australia and South America contained strains from every molecular type.

Mating type analysis: The mating type α was predominant (66 out of 74 isolates). The mating type **a** was identified from seven isolates: 2 VNIV, 1 VGI, 2 VGIII and 2 VGIV strains. Non-specific amplifications had occurred when determining the mating type **a** from the VGIII and VGIV strains. Therefore, amplicons with similar size to the mating type **a** amplicons were sequenced. BlastN search resulted in a 98% homology with the mating pheromone **a** 2 (MFa2) gene of *C. gattii* strain E566 (Genbank accession No. **AY710429**) and those strains were accordingly designated as mating type **a** (GenBank Accession No. **EU408654** – **EU408657**) (Table 1 and 3).

Phylogenetic analyses of combined loci: A heuristic search of the intron-excluded combined loci found 110 MP trees (Length 1089, CI 0.864, RI 0.984) and the intron-included combined loci analysis found 1023 MP trees (Length 1706, CI 0.866, RI 0.984). Character information and substitution models of each locus are presented in Table 4. Bayesian analyses of the combined dataset revealed topologies very similar or identical to those obtained using MP. Both intron-excluded and intron-included analyses revealed identical topologies and comparable supports for both analyses. The two currently designated species *C. neoformans* and *C. gattii* are each monophyletic with high support from both analyses. Each molecular type associated clade (VNI, VNII, VNIV, VGI, VGII, VGIII and VGIV) was also well supported using both datasets. The VNI and VNII *C. neoformans* var. *grubii* clade formed a sister relationship with the VNIV *C. neoformans* var. *neoformans* clade. The molecular type VGI, VGIII and VGIV clades are

206 closely related to each other then to the VGII clade and from as such a sister clade (*C.*
207 *gattii* a (CGa)) with the VGII clade (CGb) (Figure 1).

208 Surprisingly, three strains belonging to the molecular type VNII (M27053,
209 HamdenC3-1 and LA511) clustered with the VNI clade and not with the expected VNII
210 clade. Similarly, three strains of the molecular type VGIV (LA390, LA392 and LA568)
211 clustered with the molecular type VGIII clade. For convenience, these groups will be
212 referred to as the "VNII§ group" and the "VGIV§ group" respectively (Figure 1).

213 **Phylogenetic analyses of individual loci:** The four loci of the intron-excluded
214 and intron-included datasets contained a total of 564 and 834 parsimony informative
215 characters respectively. A hypervariable region containing poly-T was found in the
216 intron of the *PLB1* gene of some VGII strains. This region was excluded from the
217 analysis due to sequencing problems. An heuristic search of the *ACT1* gene sequences
218 found 2 MP trees (Length 351, CI 0.855, RI 0.957); the *URA5* gene found 1152 MP trees
219 (Length 208, CI 0.897, RI 0.973); the *PLB1* gene found 6 MP trees (Length 395, CI
220 0.922, RI 0.994); and the *IDE* gene found 24 MP trees (Length 113, CI 0.912, RI 0.993).
221 Character information is presented in Table 4. Bayesian analyses of the combined dataset
222 revealed topologies very similar or identical to those obtained using MP. The topologies
223 of each gene in both intron-excluded and intron-included datasets were identical or very
224 similar. Largely, the topology of each locus was similar to that of the combined datasets
225 for each molecular type clade, except for the *ACT1* locus, which showed a different
226 topology within *C. gattii*. However, all clades received high statistical support with both
227 analyses. The *C. neoformans* var. *grubii* clade showed also a sister relationship with the
228 *C. neoformans* var. *neoformans* clade. In contrast, a sister relationship between CGa

clade (VGI, VGIII and VGIV) and CGb clade (VGII) did not received consistent support across all genes (Figure 2). All strains from the VNII§ group always formed a monophyletic group which resides closely to the VNI molecular type clade, with partial VNII sequence characteristics (Figure 3). Strains from the VGIV§ group did not consistently form a monophyletic relationship with any *C. gattii* molecular type clade and did not received support from either analyses, except for the *PLB1* gene, which showed a similar topology as that of the tree of the combined loci. However, the sequence analysis revealed VGIII sequences with partial VGIV sequences integrated a similar fact as had been found in the VNII§ group for the *C. neoformans* sequences (Figure 3).

Genetic variation of each locus: Over all, the *ACT1* gene was the most conserved locus. Other loci were comparably variable. When the variation percentages were calculated for each molecular type, the VNII§ group (M27053, HamdenC3-1 and LA511) and VGIV§ group (LA390, LA392 and LA568) strains were excluded from the parsimony informative calculation due to the fact that the number of the taxa were too small to be considered in the parsimony analysis with the program PAUP. Analysis of the sequence variation found for each molecular type showed that sequences of the VNIV and VGII clades were the most variable in *C. neoformans* and *C. gattii,* respectively. Surprisingly, despite a comparable overall genetic variation of the whole species complex, the *IDE* locus was exceptionally conserved among individual molecular types compared with the other loci (Table 5). Moreover, although analysis of the combined loci revealed a comparable genetic diversity for *C. neoformans* and *C. gattii*, intra-varieties/clades variations in *C. gattii* is higher than in *C. neoformans* (Table 6).

252 **Discussions:**

253

254 The multi-gene phylogeny based on the *ACT1, URA5, PLB1* and *IDE* genes
255 confirmed the currently accepted two species concept for the *Cryptococcus* species
256 complex and supported the molecular types recognized previously by M13
257 fingerprint/AFLP/RFLP analysis. The seven clades in the phylogentic trees reflect the
258 molecular types with the exception of a few strains forming the VNII§ and VGIV§
259 groups. The sequence diversity among strains from *C. neoformans* was comparable with
260 those from *C. gattii*. Within the species *C. neoformans, C. neoformans* var. *grubii*
261 contained a 2-3 times higher level of genetic variability than that found *in C. neoformans*
262 var. *neoformans*, confirming data reported in a previous study (Boekhout et al., 2001;
263 Butler and Poulter, 2005). The MLST study of *C. neoformans* strains from sub-Saharan
264 Africa also revealed extensive genetic diversity among *C. neoformans* var. *grubii* strains
265 and proposed Southern Africa as the evolutionary origin of this species (Litvintseva et al.,
266 2003). Similar to the variation found in the four loci analyzed for *C. neoformans*, *C. gattii*
267 revealed an equivalent level of molecular variation. The four clades in the phylogenetic
268 trees corresponded to the molecular types, VGI, VGII, VGIII, and VGIV, and are highly
269 supported by both MP and Baysiean analyses. This finding are in agreement with those
270 found in South America, where extensive surveys of *Cryptococcus species* complex had
271 revealed a large genetic diversity of *C. gattii* as well as evidence of recombination
272 suggesting this area as the evolutionary origin of *C. gattii* (Escandon et al., 2006; Meyer
273 et al., 2003; Trilles et al., 2003).

274 Conflicting results between the conventional molecular typing based on PCR-
275 fingerprinting, AFLP and RFLP analysis and sequence based analysis was revealed for a
276 few strains, which formed the separate clades VNII§, closely related to the VNI calde,
277 and VGIV§, closely related to the VGIII clade. Moreover, we found that *URA5*-RFLP
278 analysis of the VNII§ and VGIV§ clades gave different molecular types from that
279 obtained by *PLB1*-RFLP analysis (data not shown). Whether recombination events or
280 genetic drift are responsible for those combination patterns of the VNII§ and VGIV§
281 strains (Figure 3) remains an open question. However, the fact that the geographic
282 locations from which the strains of the VNII§ and VGIV§ clades originated, namely
283 Africa and South America, are proposed recombination hot spots for the *Cryptococcus*
284 *species* complex (Escandon et al., 2006; Litvintseva et al., 2003; Trilles et al., 2003),
285 favor a recombination event.
286 The *ACT1* locus was conserved as it had also been observed for ascomycetous
287 yeasts, for which it was able to resolve all investigated species (Daniel and Meyer, 2003).
288 This fact was confirmed in the current study, in which it separated all molecular types of
289 the *Cryptococcus* species complex. Moreover, that study also claimed that the third codon
290 transitions are not saturated at the more recent evolutionary divergences, so all the three
291 positions could be used to infer phylogenies within a genus (Daniel and Meyer, 2003).
292 However, in the present study, it could not be totally ruled out, that the conflicting
293 topology of the *ACT1* phylogentic tree was not caused by the third codon transition
294 saturation since the divergence of *Cryptococcus* species complex (7.53%) is far more
295 than that stated in the previous study (2-4%) (Daniel and Meyer, 2003). In addition, the

296 *ACT1* phylogenetic tree became unsolvable, in fact, almost identical, after the third codon
297 was deleted (data not shown).

298 Surprisingly, despite a comparable genetic difference at the species level, the *IDE*
299 locus revealed almost no divergence within the individual molecular types. Indeed, the
300 total sequence variations were similar to that of other loci but not parsimony informative.
301 This result suggested that the *IDE* locus is a useful tool for finding a molecular cut off
302 point for speciation as dramatic increase in the genetic variation was observed from
303 molecular type to variety or species level. However, more species have to be investigated
304 in order to justify this proposal.

305 Similar to previous studies (Diaz et al., 2005; Xu et al., 2000), the current results
306 suggest a recent global dispersal of the *Cryptococcus species* complex, due to the lack of
307 geographic concordance with the phylogeny. In addition, the apparent bias of mating type
308 α over mating type **a**, as reported in previous surveys (Kwon-Chung and Bennett, 1978;
309 McClelland et al., 2004), also thwarts the yeast to undergo recombination, which in turn
310 slows down speciation. This is also emphasized by the fact that clonal populations are
311 frequently evident in several cryptococcal habitats, including Australia (Campbell et al.,
312 2005; Halliday and Carter, 2003), Thailand (Sriburee et al., 2004) and Canada (Kidd et
313 al., 2004) where the mating type **a** is scarce.

314 Recently, a new molecular type VNB was proposed as a unique population of
315 Botswana (Litvintseva et al., 2005). We conducted a preliminary parsimony analysis of
316 our *PLB1* and *URA5* data and one representative strain from each VNB sub-group
317 namely: bt1 (VNB-A), bt22 (VNB-B), bt131 (VNB) and bt31 (VNB-C) (Litvintseva et
318 al., 2005). The sequences analysis showed they are very close-related to the VNII§ clade

(data not shown). One of the strains, bt31, was almost identical to the strain M27053, which also originate from South Africa. The other two isolates, Hamden C3-1 and LA511, which originated from South America, had related but not identical sequences to bt1 and bt22, showing that those isolates are not unique to Botswana, and open up again the question for its origin. Our preliminary sequencing results suggest that the VNII§=VNB strains are most likely the result of a recombination event between the closely related molecular types VNI and VNII. However, whether VNB deserved to be a new molecular type remains an open question and further studies with more VNB strains are necessary.

In addition, a second group of strains was found in *C. gattii* VGIV§, which showed a similar recombination event as VNII§, suggesting to be the result of the recombination between VGIII and VGIV. The fact that all strains of VGIV§ originated in South America implies that recombination occurs in this part of the world. Moreover, similar to what has been found in Botswana (Litvintseva et al., 2003), a large population of mating type **a** was found in South America (Escandon et al., 2006) and those isolates are capable of mating (Escandon et al., 2007), favoring the above hypotheses.

Finally, this study provided further information on the genetic diversity among members of *Cryptococcus* species complex, showing that it consists of seven monophyletic lineages, excluding the AD hybrid (VNIII) strains. The present study highly supports the two species concept recognized currently. It clearly shows that there are four groups within the species *C. gattii*, VGI, VGII, VGIII and VGIV, which form two major phylogenetic sister clades, CGa (VGI, VGIII and VGIV) and the CGb (VGII), which are highly supported by both maximum parsimony and Bayesian analyses. A

342 similar topology was obtained previously investigating the intergenic spacer regions
343 (Diaz et al., 2005) and *PRP8* inteins (Butler and Poulter, 2005), with high support (over
344 80%) from parsimony and neighborhood-joining analysis, respectively. The molecular
345 type VGII forms the basal clade (CGb) within the *C. gattii* branch of the phylogenetic
346 tree and is phyogenetically more distant than the molecular types of the big sister calde
347 (CGa) containg VGI, VGIII and VGIV. This correlates with the finding of a specific
348 mating characteristic for the VGII Vancouver Island outbreak isolates, which produced a
349 basidium on stunted filaments close to the surface of the colonies (Fraser et al., 2003), the
350 same morphology which was also obtained by mating between other pairs of VGII
351 isolates from different parts of the world (Ngamskulrungroj and Meyer, unpublished
352 data). Finally the current study revealed a level of genetic variability among the different
353 molecular types / monophyletic lineages, which are comparable to, or in fact slightly
354 greater than that found for *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*.
355 Whether there are more than two species within the *Cryptococcus* species complex or if
356 the molecular types / monophyletic lineages within each species deserve variety status
357 remains open for discussion. Further morphological and mating studies are warranted to
358 draw final conclusions.
359
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383 **References:**
384
385 Authier, F., Bergeron, J.J., Ou, W.J., Rachubinski, R.A., Posner, B.I.,Walton, P.A. 1995
386 Degradation of the cleaved leader peptide of thiolase by a peroxisomal proteinase.
387 Proc Natl Acad Sci U S A 92: 3859-3863.

388 Boekhout, T., Theelen, B., Diaz, M., Fell, J.W., Hop, W.C., Abeln, E.C., Dromer,
389 F.,Meyer, W. 2001 Hybrid genotypes in the pathogenic yeast *Cryptococcus*
390 *neoformans*. Microbiology 147: 891-907.
391 Butler, M.I.,Poulter, R.T. 2005 The PRP8 inteins in *Cryptococcus* are a source of
392 phylogenetic and epidemiological information. Fungal Genet Biol 42: 452-463.
393 Campbell, L.T., Currie, B.J., Krockenberger, M., Malik, R., Meyer, W., Heitman,
394 J.,Carter, D. 2005 Clonality and recombination in genetically differentiated subgroups
395 of *Cryptococcus gattii*. Eukaryot Cell 4: 1403-1409.
396 Casadevall, A.,Perfect, J.R. 1998 *Cryptococcus neoformans*. ASM press, Washington,
397 D.C.
398 Chaturvedi, S., Rodeghier, B., Fan, J., McClelland, C.M., Wickes, B.L.,Chaturvedi, V.
399 2000 Direct PCR of *Cryptococcus neoformans* MATalpha and MATa pheromones to
400 determine mating type, ploidy, and variety: a tool for epidemiological and molecular
401 pathogenesis studies. J Clin Microbiol 38: 2007-2009.
402 Chen, S., Sorrell, T., Nimmo, G., Speed, B., Currie, B., Ellis, D., Marriott, D., Pfeiffer,
403 T., Parr, D.,Byth, K. 2000 Epidemiology and host- and variety-dependent
404 characteristics of infection due to *Cryptococcus neoformans* in Australia and New
405 Zealand. Australasian Cryptococcal Study Group. Clin Infect Dis 31: 499-508.
406 Chesneau, V.,Rosner, M.R. 2000 Functional human insulin-degrading enzyme can be
407 expressed in bacteria. Protein Expr Purif 19: 91-98.
408 Cox, G.M., Rude, T.H., Dykstra, C.C.,Perfect, J.R. 1995 The actin gene from
409 *Cryptococcus neoformans*: structure and phylogenetic analysis. J Med Vet Mycol 33:
410 261-266.

411 Cox, G.M., McDade, H.C., Chen, S.C., Tucker, S.C., Gottfredsson, M., Wright, L.C.,
412 Sorrell, T.C., Leidich, S.D., Casadevall, A., Ghannoum, M.A.,Perfect, J.R. 2001
413 Extracellular phospholipase activity is a virulence factor for *Cryptococcus*
414 *neoformans*. Mol Microbiol 39: 166-175.
415 Daniel, H.M.,Meyer, W. 2003 Evaluation of ribosomal RNA and actin gene sequences
416 for the identification of ascomycetous yeasts. Int J Food Microbiol 86: 61-78.
417 Diaz, M.R., Boekhout, T., Kiesling, T.,Fell, J.W. 2005 Comparative analysis of the
418 intergenic spacer regions and population structure of the species complex of the
419 pathogenic yeast *Cryptococcus neoformans*. FEMS Yeast Res 5: 1129-1140.
420 Duckworth, W.C., Bennett, R.G.,Hamel, F.G. 1998 Insulin degradation: progress and
421 potential. Endocr Rev 19: 608-624.
422 Escandon, P., Sanchez, A., Martinez, M., Meyer, W.,Castaneda, E. 2006 Molecular
423 epidemiology of clinical and environmental isolates of the *Cryptococcus neoformans*
424 species complex reveals a high genetic diversity and the presence of the molecular
425 type VGII mating type a in Colombia. FEMS Yeast Res 6: 625-635.
426 Escandon, P., Ngamskulrungroj, P., Meyer, W.,Castaneda, E. 2007 In vitro mating of
427 Colombian isolates of the *Cryptococcus neoformans* species complex. Biomedica 27:
428 308-314.
429 Espinel-Ingroff, A., Kish, C.W., Jr., Kerkering, T.M., Fromtling, R.A., Bartizal, K.,
430 Galgiani, J.N., Villareal, K., Pfaller, M.A., Gerarden, T., Rinaldi, M.G.,et al. 1992
431 Collaborative comparison of broth macrodilution and microdilution antifungal
432 susceptibility tests. J Clin Microbiol 30: 3138-3145.

433 Franzot, S.P., Fries, B.C., Cleare, W.,Casadevall, A. 1998 Genetic relationship between
434 *Cryptococcus neoformans* var. *neoformans* strains of serotypes A and D. J Clin
435 Microbiol 36: 2200-2204.
436 Fraser, J.A., Subaran, R.L., Nichols, C.B.,Heitman, J. 2003 Recapitulation of the sexual
437 cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*:
438 implications for an outbreak on Vancouver Island, Canada. Eukaryot Cell 2: 1036-
439 1045.
440 Fraser, J.A., Giles, S.S., Wenink, E.C., Geunes-Boyer, S.G., Wright, J.R., Diezmann, S.,
441 Allen, A., Stajich, J.E., Dietrich, F.S., Perfect, J.R.,Heitman, J. 2005 Same-sex mating
442 and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. Nature.
443 Garcia, J.V., Gehm, B.D.,Rosner, M.R. 1989 An evolutionarily conserved enzyme
444 degrades transforming growth factor-alpha as well as insulin. J Cell Biol 109: 1301-
445 1307.
446 Hall, T. 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis
447 program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95-98.
448 Halliday, C.L.,Carter, D.A. 2003 Clonal reproduction and limited dispersal in an
449 environmental population of *Cryptococcus neoformans* var *gattii* isolates from
450 Australia. J Clin Microbiol 41: 703-711.
451 Huelsenbeck, J.,Ronquist, F. 2001 MrBayes: Bayesian inference of phylogenetic trees.,
452 Oxford.
453 Kidd, S. 2003 Molecular Epidemiology and Characterisation of Genetic Structure to
454 Assess Speciation within the *Cryptococcus neoformans* Complex. In Medicine. Vol.
455 PhD Westmead: The University of Sydney, pp. 429.

Kidd, S.E., Hagen, F., Tscharke, R.L., Huynh, M., Bartlett, K.H., Fyfe, M., Macdougall, L., Boekhout, T., Kwon-Chung, K.J.,Meyer, W. 2004 A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc Natl Acad Sci U S A 101: 17258-17263.

Kooiman, P. 1963 The Chemical Structure of the Extracellular "Starch" Produced by *Cryptococcus Albidus* and *C. Laurentii* Var. *Flavescens*. Antonie Van Leeuwenhoek 29: 169-176.

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

Kwon-Chung, K.J.,Bennett, J.E. 1984 Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. Am J Epidemiol 120: 123-130.

Kwon-Chung, K.J., Edman, J.C.,Wickes, B.L. 1992 Genetic association of mating types and virulence in *Cryptococcus neoformans*. Infect Immun 60: 602-605.

Kwon-Chung, K.J., Boekhout, T., Fell, J.W.,Diaz, M. 2002 (1557) Proposal to conserve the name *Cryptococcus gattii* against *C.hondurianus* and *C.basillisporus* (Basidiomycota, Hymenomycetes, Tremellomycetidae). Taxon 51: 804-806.

Latouche, G.N., Huynh, M., Sorrell, T.C.,Meyer, W. 2003 PCR-restriction fragment length polymorphism analysis of the phospholipase B (PLB1) gene for subtyping of *Cryptococcus neoformans* isolates. Appl Environ Microbiol 69: 2080-2086.

Litvintseva, A.P., Marra, R.E., Nielsen, K., Heitman, J., Vilgalys, R.,Mitchell, T.G. 2003 Evidence of sexual recombination among *Cryptococcus neoformans* serotype A isolates in sub-Saharan Africa. Eukaryot Cell 2: 1162-1168.

Litvintseva, A.P., Thakur, R., Vilgalys, R.,Mitchell, T.G. 2005 Multilocus Sequence Typing Reveals Three Genetic Subpopulations of *Cryptococcus neoformans* var. *grubii* (serotype A), including a Unique Population in Botswana. Genetics.

Litvintseva, A.P., Lin, X., Templeton, I., Heitman, J.,Mitchell, T.G. 2007 Many globally isolated AD hybrid strains of *Cryptococcus neoformans* originated in Africa. PLoS Pathog 3: e114.

McClelland, C.M., Chang, Y.C., Varma, A.,Kwon-Chung, K.J. 2004 Uniqueness of the mating system in *Cryptococcus neoformans*. Trends Microbiol 12: 208-212.

Meyer, W., Mitchell, T.G., Freedman, E.Z.,Vilgalys, R. 1993 Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. J Clin Microbiol 31: 2274-2280.

Meyer, W., Marszewska, K., Amirmostofian, M., Igreja, R.P., Hardtke, C., Methling, K., Viviani, M.A., Chindamporn, A., Sukroongreung, S., John, M.A., Ellis, D.H.,Sorrell, T.C. 1999 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 20: 1790-1799.

Meyer, W., Castaneda, A., Jackson, S., Huynh, M.,Castaneda, E. 2003 Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg Infect Dis 9: 189-195.

Posada, D.,Crandall, K.A. 1998 Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817-818.

501 Samson, R.A., Stalpers, J.A.,Weijman, A.C. 1983 On the taxonomy of the entomogenous
502 fungus *Filobasidiella arachnophila*. Antonie Van Leeuwenhoek 49: 447-456.
503 Sorrell, T.C. 2001 *Cryptococcus neoformans* variety *gattii*. Med Mycol 39: 155-168.
504 Sriburee, P., Khayhan, S., Khamwan, C., Panjaisee, S.,Tharavichitkul, P. 2004 Serotype
505 and PCR-fingerprints of clinical and environmental isolates of *Cryptococcus*
506 *neoformans* in Chiang Mai, Thailand. Mycopathologia 158: 25-31.
507 Sukroongreung, S., Nilakul, C., Ruangsomboon, O., Chuakul, W.,Eampokalap, B. 1996
508 Serotypes of *Cryptococcus neoformans* isolated from patients prior to and during the
509 AIDS era in Thailand. Mycopathologia 135: 75-78.
510 Swofford, D. 2001 PAUP*. In Phylogenetic using parsimony (* and other methods)
511 Sinauer, Sunderland, MA.
512 Thompson, J.D., Higgins, D.G.,Gibson, T.J. 1994 CLUSTAL W: improving the
513 sensitivity of progressive multiple sequence alignments through sequence weighting,
514 position specific gap penalties and weight matrix choice. Nucl Acids Res 22: 4673-
515 4680.
516 Trilles, L., Lazera, M., Wanke, B., Theelen, B.,Boekhout, T. 2003 Genetic
517 characterization of environmental isolates of the *Cryptococcus neoformans* species
518 complex from Brazil. Med Mycol 41: 383-390.
519 Velegraki, A., Kiosses, V.G., Pitsouni, H., Toukas, D., Daniilidis, V.D.,Legakis, N.J.
520 2001 First report of *Cryptococcus neoformans* var. *gattii* serotype B from Greece. Med
521 Mycol 39: 419-422.

522 Xu, J., Vilgalys, R.,Mitchell, T.G. 2000 Multiple gene genealogies reveal recent
523 dispersion and hybridization in the human pathogenic fungus *Cryptococcus*
524 *neoformans*. Mol Ecol 9: 1471-1481.
525 Xu, J.,Mitchell, T.G. 2003 Comparative gene genealogical analyses of strains of serotype
526 AD identify recombination in populations of serotypes A and D in the human
527 pathogenic yeast *Cryptococcus neoformans*. Microbiology 149: 2147-2154.
528

Figure legends:

Figure 1. Combined genealogies of (A) the intron-excluded and (B) the intron-included datasets with separate substitution models for each partition. Parsimony bootstrap support above 75 is bold. Bayesian posterior probability above 95 is italicized. The phylogenetic tree is rooted using *Filobasidiella depauperata* and *Cryptococcus albidus* as outgroups. The blue bold italic letters represent VNII§ and VGIV§ clades of *C. neoformans* and *C. gattii* respectively.

Figure 2. Gene genealogies of four individual loci genearted by Maximum Parsimony analysis. Parsimony bootstrap support above 75 is bold. Bayesian posterior probability above 95 is italicized. Phylogenetic trees are unrooted. The blue bold italic letters represent VNII§ and VGIV§ clades of *C. neoformans* and *C. gattii* respectively.

Figure 3. *URA5* and *PLB1* alignments of the VNII§ and VGIV§ strains revealed sequence similarity to VNI (ATCC90112 and WM148) and VGIII (WM175 and CN043) respectively. However, some parts of the sequence were similar to that of VNII (WM626 and RV58146) for the VNII§ strains and VGIV (WM779 and M27056) for the VGIV§ strains (shaded area).

Figure 1

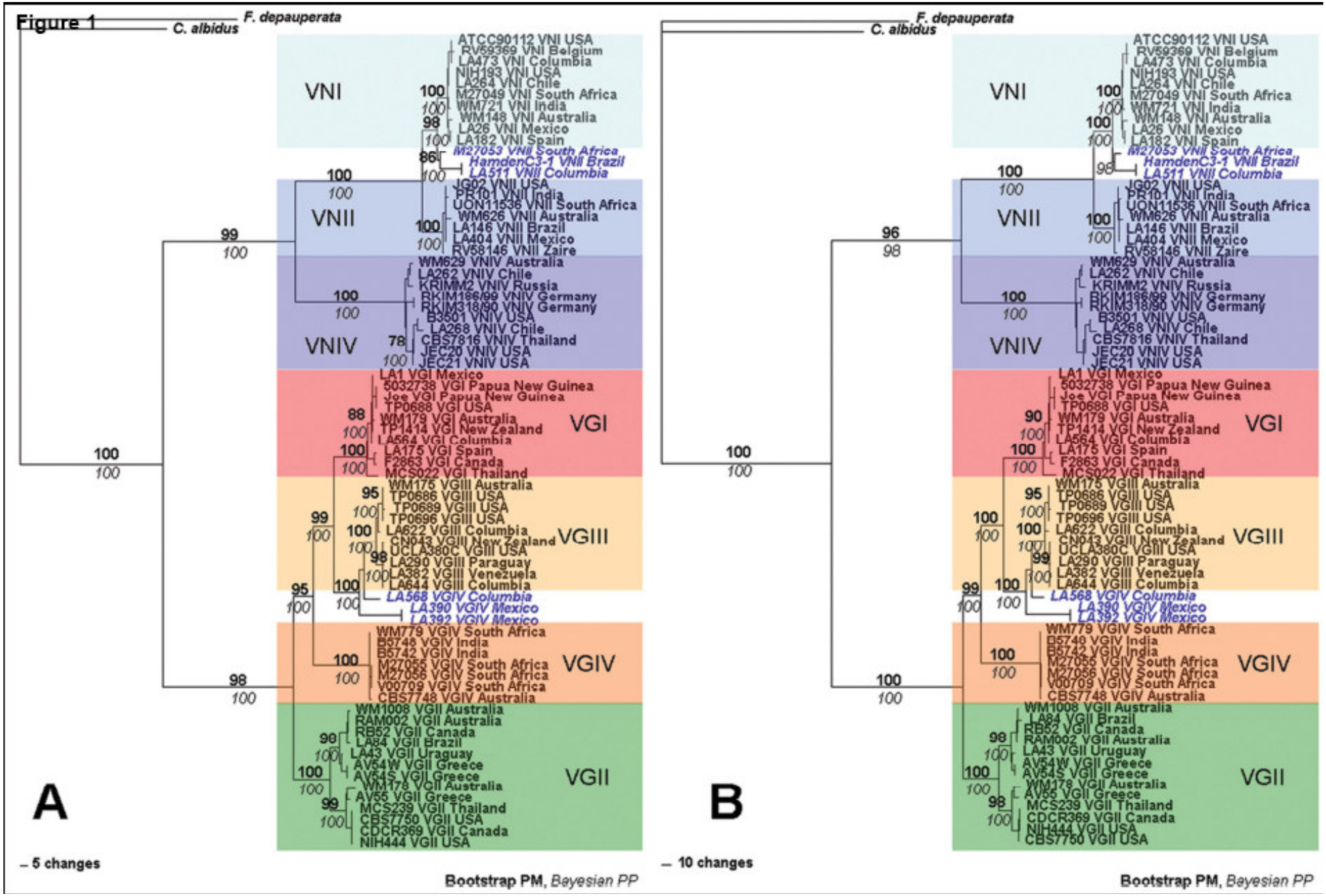

F. depauperata
C. albidus
VNI
VNII
VNIV
VGI
VGIII
VGIV
VGII
ATCC90112 VNI USA
RV59369 VNI Belgium
LA473 VNI Columbia
NIH193 VNI USA
LA264 VNI Chile
M27049 VNI South Africa
WM721 VNI India
WM148 VNI Australia
LA26 VNI Mexico
LA182 VNI Spain
M27053 VNII South Africa
HamdenC3-1 VNII Brazil
LA511 VNII Columbia
JG02 VNII USA
PR101 VNII India
UON11536 VNII South Africa
WM626 VNII Australia
LA146 VNII Brazil
LA404 VNII Mexico
RV58146 VNII Zaire
WM629 VNIV Australia
LA262 VNIV Chile
KRIMM2 VNIV Russia
RKIM186/99 VNIV Germany
RKIM318/90 VNIV Germany
B3501 VNIV USA
LA268 VNIV Chile
CBS7816 VNIV Thailand
JEC20 VNIV USA
JEC21 VNIV USA
LA1 VGI Mexico
5032738 VGI Papua New Guinea
Joe VGI Papua New Guinea
TP0688 VGI USA
WM179 VGI Australia
TP1414 VGI New Zealand
LA564 VGI Columbia
LA175 VGI Spain
F2863 VGI Canada
MCS022 VGI Thailand
WM175 VGIII Australia
TP0686 VGIII USA
TP0689 VGIII USA
TP0696 VGIII USA
LA622 VGIII Columbia
CN043 VGIII New Zealand
UCLA380C VGIII USA
LA290 VGIII Paraguay
LA382 VGIII Venezuela
LA644 VGIII Columbia
LA568 VGIV Columbia
LA390 VGIV Mexico
LA392 VGIV Mexico
WM779 VGIV South Africa
B5740 VGIV India
B5742 VGIV India
M27055 VGIV South Africa
M27056 VGIV South Africa
V00709 VGIV South Africa
CBS7748 VGIV Australia
WM1008 VGII Australia
RAM002 VGII Australia
RB52 VGII Canada
LA84 VGII Brazil
LA43 VGII Uruguay
AV54W VGII Greece
AV54S VGII Greece
WM178 VGII Australia
AV55 VGII Greece
MCS239 VGII Thailand
CBS7750 VGII USA
CDCR369 VGII Canada
NIH444 VGII USA
A
– 5 changes
Bootstrap PM, Bayesian PP
B
– 10 changes
Bootstrap PM, Bayesian PP

Figure 2

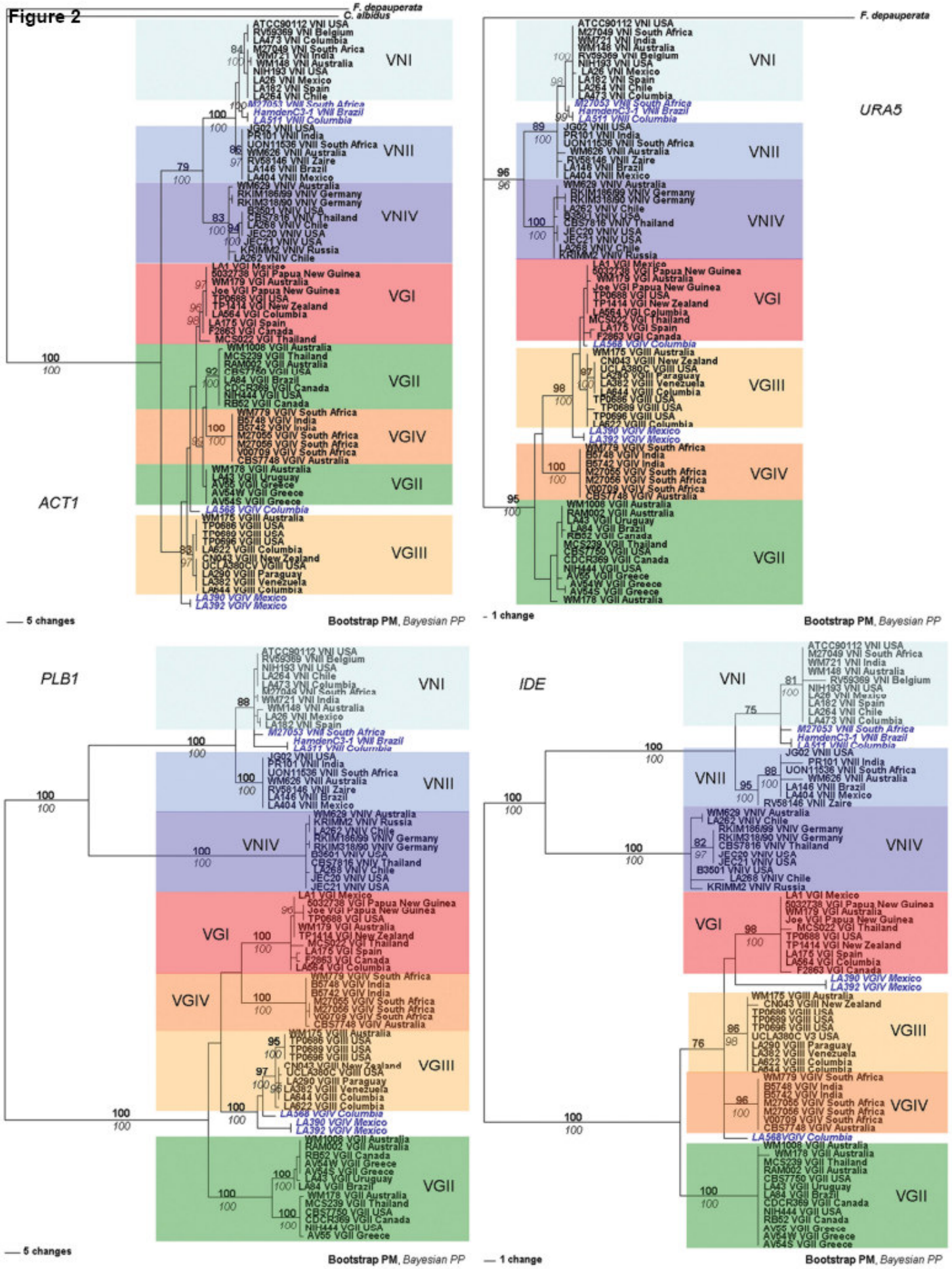
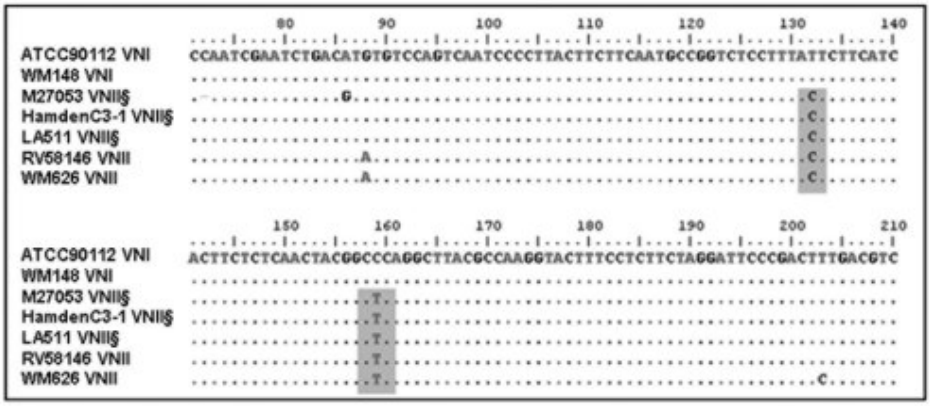

F. depauperata
C. albidus
ACTC90112 VNI USA
RV59369 VNI Belgium
LA473 VNI Columbia
M27049 VNI South Arica
WM721 VNI India
WM148 VNI Australia
NIH193 VNI USA
LA26 VNI Mexico
LA182 VNI Spain
LA264 VNI Chile
M27053 VNII South Africa
HamdenC3-1 VNII Brazil
LA511 VNII Columbia
JG02 VNII USA
PR101 VNII India
UON11536 VNII South Africa
WM626 VNII Australia
RV58146 VNII Zaire
LA146 VNII Brazil
LA404 VNII Mexico
WM629 VNIV Australia
RKIM186/99 VNIV Germany
RKIM318/90 VNIV Germany
B3501 VNIV USA
CBS7816 VNIV Thailand
LA268 VNIV Chile
JEC20 VNIV USA
JEC21 VNIV USA
KRIMM2 VNIV Russia
LA262 VNIV Chile
LA1 VGI Mexico
5032738 VGI Papua New Guinea
WM179 VGI Australia
Joe VGI Papua New Guinea
TP0688 VGI USA
TP1414 VGI New Zealand
LA564 VGI Columbia
LA175 VGI Spain
F2863 VGI Canada
MCS022 VGI Thailand
WM1008 VGII Australia
MCS239 VGII Thailand
RAM002 VGII Australia
CBS7750 VGII USA
LA84 VGII Brazil
CDCR369 VGII Canada
NIH444 VGII USA
RB52 VGII Canada
WM779 VGIV South Africa
B5748 VGIV India
B5742 VGIV India
M27055 VGIV South Africa
M27056 VGIV South Africa
V00709 VGIV South Africa
CBS7748 VGIV Australia
WM178 VGII Australia
LA43 VGII Uruguay
AV55 VGII Greece
AV54W VGII Greece
AV54S VGII Greece
LA568 VGIV Columbia
WM175 VGIII Australia
TP0686 VGIII USA
TP0689 VGIII USA
TP0696 VGIII USA
LA622 VGIII Columbia
CN043 VGIII New Zealand
UCLA380CV VGIII USA
LA290 VGIII Paraguay
LA382 VGIII Venezuela
LA644 VGIII Columbia
LA390 VGIV Mexico
LA392 VGIV Mexico
VNI
VNII
VNIV
VGI
VGII
VGIII
ACT1
5 changes
Bootstrap PM, Bayesian PP
F. depauperata
URA5
1 change
Bootstrap PM, Bayesian PP
PLB1
5 changes
Bootstrap PM, Bayesian PP
IDE
1 change
Bootstrap PM, Bayesian PP

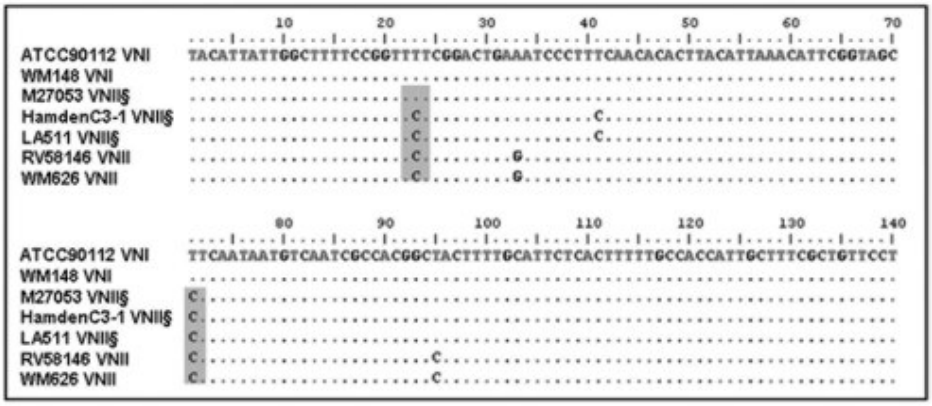
Figure 3

C. neoformans

URA5

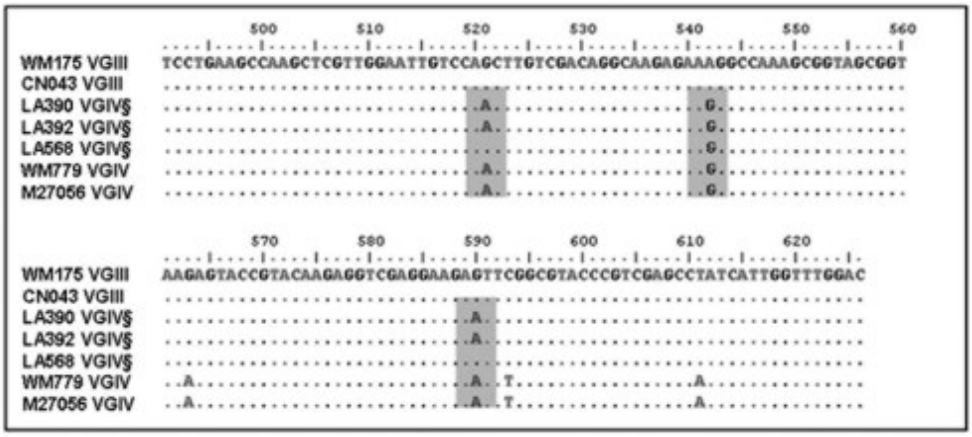


PLB1



C. gattii

URA5



PLB1

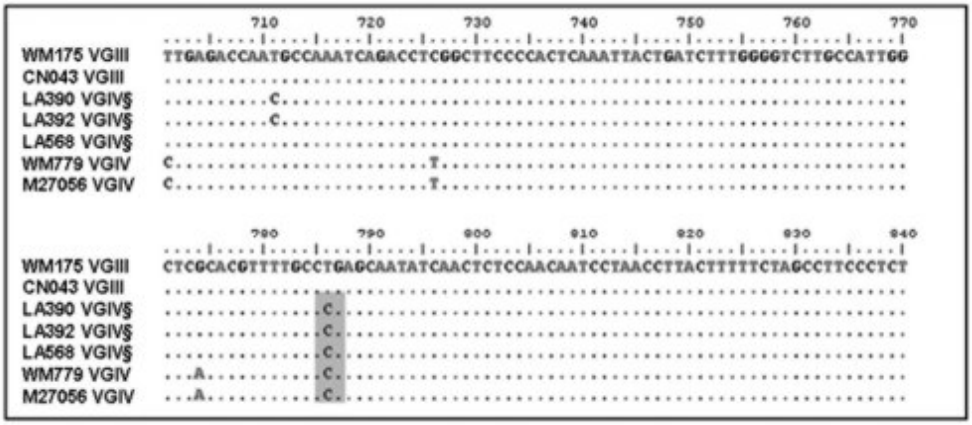


Table 1

Table 1. Strains used in this study.

Name	Country	Source	Serotype	MAT	Molecular type	Species name	Variety	References
CBS7841	Canada	ENV	NA	NA	NA	*Filobasidiella depauperata*	NA	(Samson et al., 1983)
CBS142	Japan	ENV	NA	NA	NA	*Cryptococcus albidus*	NA	(Kooiman, 1963)
ATCC 90112	USA	CLIN	A	alpha	VNI	*Cryptococcus neoformans*	*grubii*	(Espinel-Ingroff et al., 1992)
M27049	South Africa	CLIN	-	alpha	VNI	*Cryptococcus neoformans*	*grubii*	This study
WM 721	India	ENV	A	alpha	VNI	*Cryptococcus neoformans*	*grubii*	This study
WM 148^{R}	Australia	CLIN	A	alpha	VNI	*Cryptococcus neoformans*	*grubii*	(Meyer et al., 2003)
RV 59369	Belgium	ENV	A	alpha	VNI	*Cryptococcus neoformans*	*grubii*	This study
NIH 193	USA	ENV	A	alpha	VNI	*Cryptococcus neoformans*	*grubii*	This study
LA 26	Mexico	ENV	A	alpha	VNI	*Cryptococcus neoformans*	*grubii*	(Meyer et al., 2003)
LA 182	Spain	CLIN	A	alpha	VNI	*Cryptococcus neoformans*	*grubii*	(Meyer et al., 2003)
LA 264	Chile	CLIN	A	alpha	VNI	*Cryptococcus neoformans*	*grubii*	(Meyer et al., 2003)
LA 473	Columbia	CLIN	A	alpha	VNI	*Cryptococcus neoformans*	*grubii*	(Meyer et al., 2003)
JG-02	USA	CLIN	-	alpha	VNII	*Cryptococcus neoformans*	*grubii*	This study
M27053	South Africa	CLIN	A	alpha	VNII	*Cryptococcus neoformans*	*grubii*	This study

PR-101	India	CLIN	-	alpha	VNII	*Cryptococcus neoformans*	*grubii*	This study
UON 11536	South Africa	CLIN	-	alpha	VNII	*Cryptococcus neoformans*	*grubii*	This study
WM626^{R}	Australia	CLIN	A	alpha	VNII	*Cryptococcus neoformans*	*grubii*	(Meyer et al., 2003)
Hamden C3-1	Brazil	ENV	A	alpha	VNII	*Cryptococcus neoformans*	*grubii*	(Boekhout et al., 2001)
RV 58146	Zaire	ENV	A	alpha	VNII	*Cryptococcus neoformans*	*grubii*	(Diaz et al., 2005)
LA 146	Brazil	ENV	A	alpha	VNII	*Cryptococcus neoformans*	*grubii*	(Meyer et al., 2003)
LA 404	Mexico	CLIN	A	alpha	VNII	*Cryptococcus neoformans*	*grubii*	(Meyer et al., 2003)
LA 511	Columbia	CLIN	A	alpha	VNII	*Cryptococcus neoformans*	*grubii*	(Meyer et al., 2003)
WM 629^{R}	Australia	CLIN	D	alpha	VNIV	*Cryptococcus neoformans*	*neoformans*	(Meyer et al., 2003)
RKI-M186/99	Germany	CLIN	D	alpha	VNIV	*Cryptococcus neoformans*	*neoformans*	This study
RKI-M318/90	Germany	CLIN	D	alpha	VNIV	*Cryptococcus neoformans*	*neoformans*	This study
B-3501	USA	CLIN	D	alpha	VNIV	*Cryptococcus neoformans*	*neoformans*	(Meyer et al., 1993)
CBS 7816	Thailand	ENV	D	a	VNIV	*Cryptococcus neoformans*	*neoformans*	(Diaz et al., 2005)
LA268	Chile	CLIN	D	alpha	VNIV	*Cryptococcus neoformans*	*neoformans*	(Meyer et al., 2003)
JEC 20	USA	NA	D	a	VNIV	*Cryptococcus neoformans*	*neoformans*	(Kwon-Chung et al., 1992)
JEC 21	USA	NA	D	alpha	VNIV	*Cryptococcus neoformans*	*neoformans*	(Kwon-Chung et al.,

								1992)
KRIMM 2	Russia	CLIN	-	alpha	VNIV	*Cryptococcus neoformans*	*neoformans*	This study
LA262	Chile	CLIN	D	alpha	VNIV	*Cryptococcus neoformans*	*neoformans*	(Meyer et al., 2003)
LA1	Mexico	CLIN	B	alpha	VGI	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
503 2738	Papua New Guinea	CLIN	B	alpha	VGI	*Cryptococcus gattii*	NA	(Chen et al., 2000)
WM179^{R}	Australia	CLIN	B	alpha	VGI	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
Joe	Papua New Guinea	CLIN	B	alpha	VGI	*Cryptococcus gattii*	NA	(Chen et al., 2000)
MC-S-022	Thailand	CLIN	B	alpha	VGI	*Cryptococcus gattii*	NA	(Sukroongreung et al., 1996)
TP 0688	USA	ENV	B	alpha	VGI	*Cryptococcus gattii*	NA	This study
TP 1414	New Zealand	VET	B	alpha	VGI	*Cryptococcus gattii*	NA	This study
LA175	Spain	CLIN	B	alpha	VGI	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
LA564	Columbia	CLIN	B	alpha	VGI	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
F 2863	Canada	VET	B	alpha	VGI	*Cryptococcus gattii*	NA	(Kidd et al., 2004)
WM1008	Australia	ENV	-	alpha	VGII	*Cryptococcus gattii*	NA	(Chen et al., 2000)
WM178^{R}	Australia	CLIN	B	alpha	VGII	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
MC-S-239	Thailand	CLIN	B	alpha	VGII	*Cryptococcus gattii*	NA	(Sukroongreung et al., 1996)

RAM 002	Australia	ENV	-	alpha	VGII	*Cryptococcus gattii*	NA	(Chen et al., 2000)
CBS 7750	USA	ENV	B	alpha	VGII	*Cryptococcus gattii*	NA	(Kidd et al., 2004)
LA43	Uruguay	ENV	B	alpha	VGII	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
LA84	Brazil	CLIN	-	alpha	VGII	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
CDC R369	Canada	CLIN	B	alpha	VGII	*Cryptococcus gattii*	NA	(Kidd et al., 2004)
NIH 444	USA	CLIN	B	alpha	VGII	*Cryptococcus gattii*	NA	(Fraser et al., 2005)
RB52	Canada	ENV	B	alpha	VGII	*Cryptococcus gattii*	NA	(Kidd et al., 2004)
AV 55	Greece	CLIN	B	a	VGII	*Cryptococcus gattii*	NA	(Velegraki et al., 2001)
AV 54W	Greece	CLIN	B	alpha	VGII	*Cryptococcus gattii*	NA	(Velegraki et al., 2001)
AV 54S	Greece	CLIN	B	alpha	VGII	*Cryptococcus gattii*	NA	(Velegraki et al., 2001)
WM175^{R}	Australia	CLIN	B	alpha	VGIII	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
CN043	New Zealand	CLIN	-	a	VGIII	*Cryptococcus gattii*	NA	(Chen et al., 2000)
TP 0686	USA	ENV	B	alpha	VGIII	*Cryptococcus gattii*	NA	This study
TP 0689	USA	ENV	B	alpha	VGIII	*Cryptococcus gattii*	NA	This study
TP 0696	USA	ENV	B	alpha	VGIII	*Cryptococcus gattii*	NA	This study

UCLA 380C	USA	N	C	alpha	VGIII	*Cryptococcus gattii*	NA	(Meyer et al., 1993)
LA 290	Paraguay	CLIN	-	alpha	VGIII	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
LA 382	Venezuela	CLIN	C	alpha	VGIII	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
LA 622	Columbia	CLIN	B	a	VGIII	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
LA 644	Columbia	ENV	C	alpha	VGIII	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
WM 779^{R}	South Africa	VET	C	alpha	VGIV	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
B-5748	India	CLIN	B	alpha	VGIV	*Cryptococcus gattii*	NA	This study
B-5742	India	CLIN	B	alpha	VGIV	*Cryptococcus gattii*	NA	This study
M27055	South Africa	CLIN	C	alpha	VGIV	*Cryptococcus gattii*	NA	This study
M27056	South Africa	CLIN	-	alpha	VGIV	*Cryptococcus gattii*	NA	This study
V00709	South Africa	CLIN	C	alpha	VGIV	*Cryptococcus gattii*	NA	This study
CBS 7748	Australia	ENV	B	alpha	VGIV	*Cryptococcus gattii*	NA	(Diaz et al., 2005)
LA 390	Mexico	CLIN	-	a	VGIV	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
LA 392	Mexico	CLIN	-	a	VGIV	*Cryptococcus gattii*	NA	(Meyer et al., 2003)
LA 568	Columbia	CLIN	B	alpha	VGIV	*Cryptococcus gattii*	NA	(Meyer et al., 2003)

- = unknown, NA = not applicable, CLIN = Clinical, ENV = Environmental, VET = Veterinary, R = molecular type reference strains

Table 2. Primers used in this study

Primers names	Primers sequences	Note	Source
CNACT1	5' AATCTCGCCCAACATGT 3'	Amplify *ACT1*	This study
CNACT1R	5' TTAGAAACACTTTCGGTGGACG 3'	Amplify *ACT1*	This study
CNACT1F2	5' CCAAGCAGAACCGAGAGAAG 3'	Internal primers of *ACT1*	This study
URA5	5' ATGTCCTCCCAAGCCCTCGACTCCG 3'	Amplify *URA5*	(Meyer et al., 2003)
SJ101	5' TTAAGACCTCTGAACACCGTACTC 3'	Amplify *URA5*	(Meyer et al., 2003)
PLBCNAF	5' TAAAGTGCTTGGTGGGAACC 3'	Amplify *PLB1* from VN	This study
PLBCNAR	5' TCTCGCGAGGATTACAGGAT 3'	Amplify *PLB1* from VN	This study
PLBCG2F	5' TCCCCTTCAACACAGCTCTT 3'	Amplify *PLB1* from VG	This study
PLBCG2R2	5' CACCTATCTTCGCTGCATCA 3'	Amplify *PLB1* from VG	This study
PLBCNIF1	5' GGTTACCGTGCAATGCTGT 3'	Internal primers of *PLB1*	This study
PLBCNIF2	5' GGTGCTTTCACCCCTATTGA 3'	Internal primers of *PLB1*	This study
PLBCNIR1	5' CGGGAAATATCAGCTTGGTC 3'	Internal primers of *PLB1*	This study
IDEF	5' CCAAGGCGGACAAGGCTGCGG 3'	Amplify *IDE*	This study
IDER	5' GTAGAGGTGATCCATGTCGGG 3'	Amplify *IDE*	This study
ACT1CAF1	5' GGTGTCATGGTCGGTATGG 3'	Amplify *ACT1* from CA	This study

ACT1CAR1	5' GTACTTTCGCTCGGGAGGAG 3'	Amplify *ACT1* from CA	This study
ACT1CAR2	5' AGCTTCTCCTTGATGTCTC 3'	Amplify *ACT1* from CA	This study
URA5DF1	5' CCWTACTTCTTCAAYGCYGG 3'	Amplify *URA5* from FD	This study
MFLL	5' CTTCACTGCCATCTTCACCA 3'	Mating type **α** determination of VN	(Chaturvedi et al., 2000)
MFLR	5' GACACAAAGGGTCATGCCA 3'	Mating type **α** determination of VN	(Chaturvedi et al., 2000)
MFAL	5' CGCCTTCACTGCTACCTTCT 3'	Mating type **a** determination of VN	(Chaturvedi et al., 2000)
MFAR	5' AACGCAAGAGTAAGTCGGGC 3'	Mating type **a** determination of VN	(Chaturvedi et al., 2000)
MF**α**U	5' TTCACTGCCATCTTCACCACC 3'	Mating type **α** determination of VG	(Halliday and Carter, 2003)
MF**α**L	5' TCTAGGCGATGACACAAAGGG 3'	Mating type **α** determination of VG	(Halliday and Carter, 2003)
MFa2U	5' ACACCGCCTGTTACAATGGAC 3'	Mating type **a** determination of VG	(Fraser et al., 2003)
MFa2L	5' CAGCGTTTGAAGATGGACTTT 3'	Mating type **a** determination of VG	(Fraser et al., 2003)

VN = molecular type VN1,2 and 3; VG = molecular type VG1, 2, 3 and 4, FD = *Filobasidiella depauperata*, CA= *Cryptococcus albidus*

Table 3. Geographic and mating type data.

Molecular type	Continent						Total*
	Africa	Asia	Australia	Europe	North America	South America	
VNI	1	1	1	2	2	3	10 (0)
VNII	3	1	1	0	1	4	10 (0)
VNIV	0	1 (1)		3	3 (1)	2	10 (2)
VGI	0	1	4	1	2	2	10 (0)
VGII	0	1	3	3 (1)	4	2	13 (1)
VGIII	0	0	2 (1)	0	4	4 (1)	10 (2)
VGIV	4	2	1	0	0	3 (2)	10 (2)
Total	8	9	14	15	16	21	83 (7)

* Numbers in bracket represent the number of mating type **a** strains of each molecular type.

Table 4. The character of each locus and combined data.

Locus	Character (intron-excluded)			Character (intron-included)			substitution model
	total	constant	parsimony informative	total	constant	parsimony informative	
ACT1	1124	848	177	1321	808	187	GTR+G
URA5	621	457	71	724	509	102	SYM+G
PLB1	1877	1549	309	2265	1819	423	HKY+G
IDE	581	483	85	684	549	122	K80+G*
Combined	4203	3337	564	4994	3685	834	N/A

N/A = not applicable (partition of the dataset was used), * The second best chosen by the ModelTest program since the best one TrN+I and TrN+G could not be operated due to the limitation in number of nucleotide substitution type could be performed MrBayes program.

Table 5

Table 5. Genetic variation among the molecular types of *Cryptococcus species* complex.

Locus		*ACT1*		*URA5*		*PLB1*		*IDE*	
Total Characters		***1062***		***525***		***1877***		***581***	
Variable characters		**%Total**	**%PI**	**%Total**	**%PI**	**%Total**	**%PI**	**%Total**	**%PI**
C. neoformans	VNI	0.28	0.28	0.19	0.19	0.32	0.21	0.34	0.00
	VNII	0.00	0.00	0.57	0.00	0.00	0.00	1.03	0.00
	VNII§	0.00	N/A	0.00	N/A	0.01	N/A	0.00	N/A
	VNIV	0.75	0.47	0.76	0.76	0.16	0.11	1.20	0.52
C. gattii	VGI	0.47	0.09	0.95	0.57	0.64	0.32	0.00	0.00
	VGII	0.66	0.56	1.71	0.57	1.12	0.96	0.17	0.00
	VGIII	0.19	0.19	0.76	0.38	0.37	0.21	0.17	0.00
	VGIV	0.00	0.00	0.00	0.00	0.11	0.05	0.00	0.00
	VGIV§	0.01	N/A	0.01	N/A	0.01	N/A	0.02	N/A
Mean*		**0.34**	**0.23**	**0.71**	**0.35**	**0.39**	**0.27**	**0.42**	**0.07**
C.s.* complex*		**7.53**	**6.97**	**15.43**	**13.33**	**17.47**	**16.46**	**16.87**	**14.63**

Note: The VNII§ and VGIV§ PI were excluded due to limitation of the PAUP program. (*Mean = mean of variable percentage of all original molecular types. ***C.s.* complex = variable percentage calculated from the whole datasets. PI = parsimony informative. N/A = not applicable)

Table 6. Number of polymorphic sites of the intron-excluded combined dataset among different clades of the *Cryptococcus species* complex

Species and clades	Molecular type	No. of polymorphic characters	No. of parsimony informative characters
C. neoformans		255	233
var. *grubii* clade		73	57
	VNI	12	7
	VNII*	9	0
var. *neoformans* clade	VNIV	22	14
C. gattii		236	201
CGa clade		181	153
	VGI	24	10
	VGIII	14	8
	VGIV*	2	1
CGb clade	(VGII)	38	27

• VNII§ and VGIV§ was excluded

4.2 Análise morfológica comparativa

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4.2.1 An alternative method to prepare samples of the pathogenic yeast *Cryptococcus neoformans* for scanning electron microscopy analysis

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Note

An alternative method to prepare samples of the pathogenic yeast *Cryptococcus neoformans* for scanning electron microscopy analysis

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Abstract

In this work, an alternative to conventional preparation procedures for scanning electron microscopy (SEM) analysis of *Cryptococcus neoformans* was performed. The cells were fixed directly in the agar culture. This method is simpler than others already reported and the morphology of the cells was well preserved.

Keywords: SEM; Electron microscopy; *Cryptococcus neoformans*; Morphology; Fixation; Dehydration

Scanning electron microscopy (SEM) has been used in order to study biochemical and morphological growth characteristics of *Cryptococcus neoformans* (Cleare and Casadevall, 1999; Nosanchuk et al., 1999). *C. neoformans* is an encapsulated pathogenic fungus that causes life-threatening meningoencephalitis, particularly in immunocompromised individuals. This yeast has a polysaccharide capsule composed of 80% glucuroxylomannan (GXM), its major antigenic component which has been used to classify cryptococcal strains into serotypes (Cleare and Casadevall, 1999; Janbon, 2004). Currently, three varieties are recognized: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), and *C. neoformans* var. *gattii* (serotypes B and C), but this classification has still been discussed (Franzot et al., 1999; Katsu et al., 2004). The presence of the capsule is associated with virulence and it is the main virulence factor. The capsular polysaccharide has been implicated in a variety of potentially deleterious

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effects, including inhibition of phagocytosis, induction of brain edema, complement activation and cytokine deregulation (Cleare and Casadevall, 1999; Janbon, 2004). The capsule is found immediately outside the cell wall and may vary in size from <1 to >50 μm, depending on the strain, environment, and growth conditions (Casadevall and Perfect, 1998). SEM has been useful in the investigation of the capsular polysaccharide characteristics. Moreover, it was used to analyze the shapes of the cells and the state of the polysaccharide capsule in the presence of antifungal drugs (Casadevall and Perfect, 1998; Cleare and Casadevall, 1999; Nosanchuk et al., 1999).

The usual procedures for SEM analysis involve the fixation of a dense layer of cells to polylysine-coated slides before the dehydration, which can be achieved either by chemical dehydration or freeze-drying (Cleare and Casadevall, 1999). This method is time-consuming or expensive due to the necessity of polylysine-coated slides. Any approach for less-laborious sample preparation causes less morphological and chemical changes to biological samples and if it is less expensive, it would be of much value to scanning electron microscopy.

In this work we report an alternative to conventional preparation procedures for scanning electron microscopy (SEM) analysis of *C. neoformans*. The samples are prepared directly in the agar culture instead of the use of polylysine-coated slides. Clinical isolates of *C. neoformans*, HC6 (var. *grubii*, serotype A) and AL33 (var. *gattii*, serotype B), were used in this work. The strains were kindly provided by Hospital de Clínicas de Porto Alegre, Porto Alegre, RS and Hospital Santa Casa de Misericórdia, Porto Alegre, RS, respectively, and previously characterized by molecular and biochemical methods (Casali et al., 2003; Horta et al., 2002). The serotype of the strains was determined by the slide agglutination test (IATRON Crypto Check, Iatron, Tokyo, Japan) according to the manufacturers' instructions.

The isolates of *C. neoformans* were cultured in Sabouraud dextrose agar (SDA) for 9 h, potato agar dextrose or birdseed agar — *Guizotia abyssinica* (Casadevall and Perfect, 1998), for 48 h at 30 °C

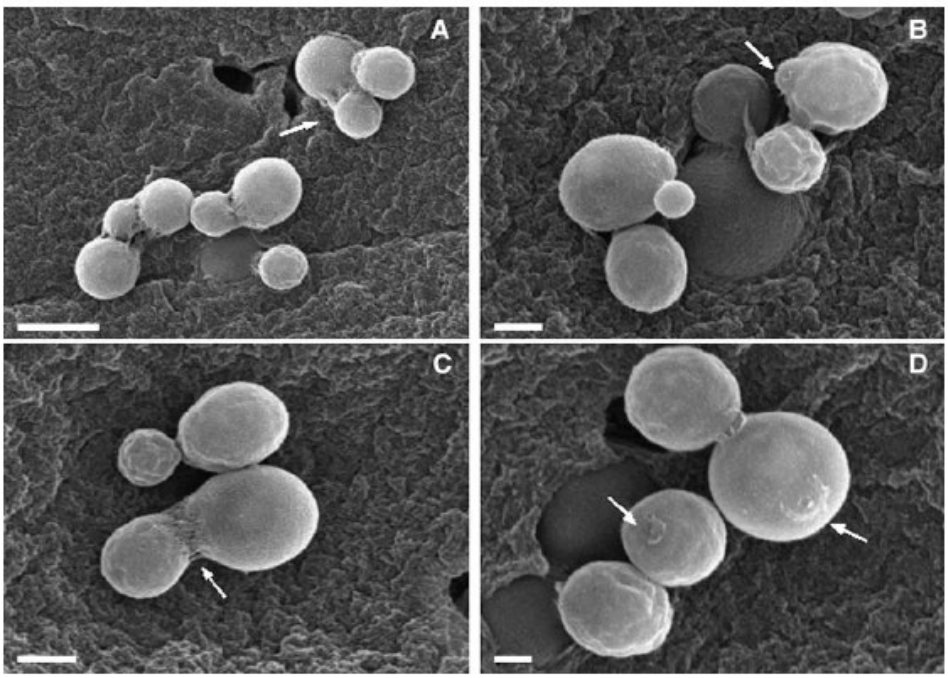

Fig. 1. SEM of *C. neoformans* in SDA agar. The budding formation in A, B and C and bud scars in D is indicated by an arrow. (A) Bar: 5 μm. (B) Bar: 2 μm. (C) Bar: 2 μm. (D) Bar: 1 μm. The yeasts were fixed after 9 h of culture at 30 °C.

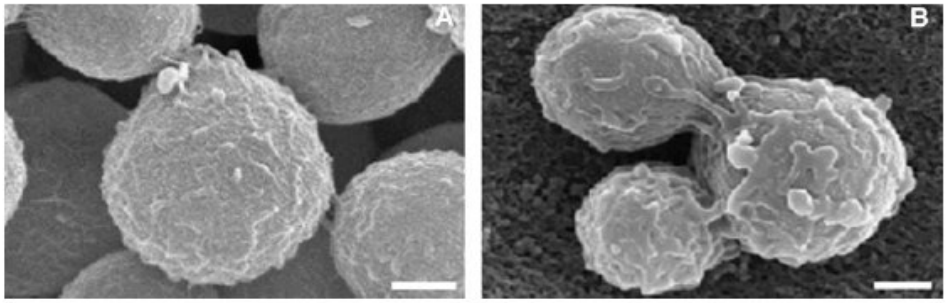

Fig. 2. SEM of *C. neoformans* var. *grubii* and *C. neoformans* var. *gattii* in potato dextrose agar. (A) *Cryptococcus neoformans* var. *grubii* (HC6) and (B) *Cryptococcus neoformans* var. *gattii* (AL33). Bar: 1 μm. The yeasts were fixed after 48 h of culture at 30 °C.

and small blocks of agar with culture were collected for fixation. The samples were fixed overnight at 4 °C with 2% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. Post-fixation was carried out in 1% (w/v) osmium tetroxide in the same buffer. The specimens were rinsed in buffer, dehydrated in a series of 30–100% acetone solutions, dried at critical point in CO_2 (CPD 030 BALTEC) and coated with gold in a sputter-coater (SCD 050 BALTEC). The material was examined in a Jeol JSM 5800 scanning electron microscope (SEM) at the Centro de Microscopia Eletrônica da Universidade Federal do Rio Grande do Sul — CME/UFRGS, Porto Alegre/RS at an accelerating voltage of 20 kV.

C. neoformans cells were globular and ranged from 2 to 8 μm in diameter. When the cells of *C. neoformans* var. *grubii* were cultured in SDA agar, SEM images showed globular cells with the surface relatively smooth except for scattered small protrusions. These protrusions are bud scars of <1 μm in diameter as observed on Fig. 1.

In order to study the possible differences between the morphology of both varieties of *C. neoformans*, i.e. var. *grubii* and var. *gattii*, we observed the isolates HC6 and AL33 after 48 h of culture in potato dextrose agar, which stimulates capsule formation. Moreover, the same isolates were evaluated in three different agar cultures (birdseed, potato dextrose and SDA) also after 48 h. The strains appear covered with a

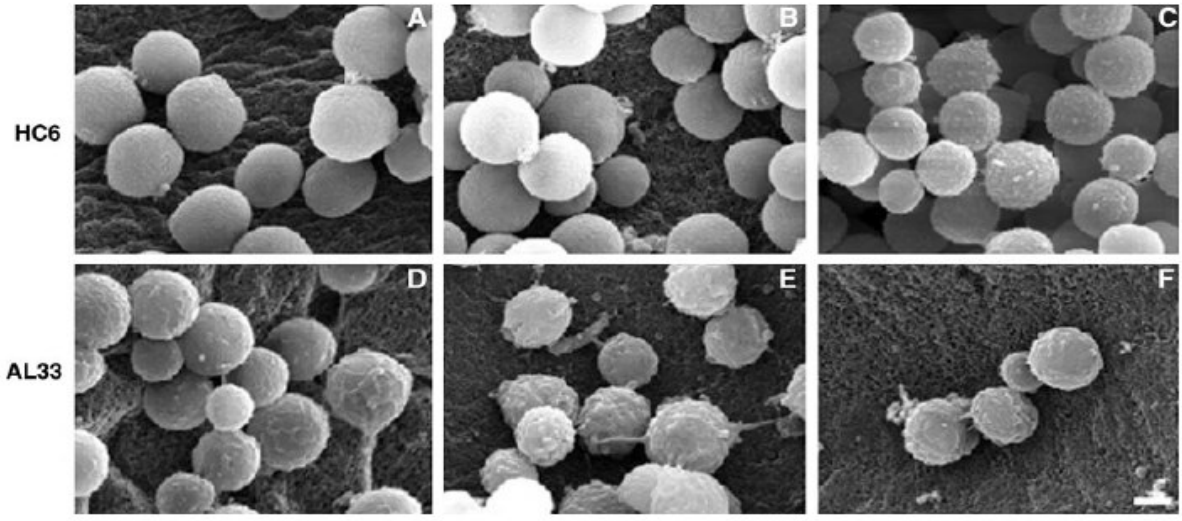

Fig. 3. SEM of *C. neoformans* var. *grubii* and *C. neoformans* var. *gattii* in three different agar cultures. (A and D) *Cryptococcus neoformans* var. *grubii* (HC6) and *Cryptococcus neoformans* var. *gattii* (AL33) in SDA agar; (B and E) in birdseed agar and (C and F) in potato dextrose agar. Bar: 2 μm. The yeasts were fixed after 48 h of culture at 30 °C.

mucoid substance, probably the polysaccharide capsule. Different morphological aspects in potato dextrose agar were observed between the varieties. In potato dextrose agar *C. neoformans* var. *gattii* presented a more apparent capsule when compared to var. *grubii* (Fig. 2). The isolate of *C. neoformans* var. *grubii* is stimulated to produce capsule in potato dextrose agar, while var. *gattii* produces polysaccharide capsule in any agar culture (Fig. 3). SEM analysis in SDA and birdseed agar revealed ovoid cells without mucoid aspect to *C. neoformans* var. *grubii*. In accordance with literature, *C. neoformans* var. *gattii* differs from *C. neoformans* var. *grubii* in a number of physiological characteristics. It is possible that the presence of the pronounced polysaccharide capsule would be related with a higher patogenicity of *C. neoformans* var. *gattii* (Sorrell, 2001). Moreover, colonies of *C. neoformans* var. *gattii* appear highly mucoid when compared with *C. neoformans* var. *grubii*.

In a previous study, the SEM appearance of *C. neoformans* cells was shown to depend on several variables, including dehydration protocol, protein binding, and age of culture (Cleare and Casadevall, 1999). Other studies have demonstrated that when evaluating electron micrographs of the capsule, it is important to consider that capsules are usually dehydrated for microscopy, and drying artifacts may occur. Moreover, the relevance of the capsular structural features observed in dehydrated cells by electron microscopy techniques to the normal hydrated state is unknown (Casadevall and Perfect, 1998).

The appearance of cells during budding has been visualized, and our studies confirm that bud scars were easily discernible on the surface of non-encapsulated cells (Cleare and Casadevall, 1999). The results suggest that this method is simpler than others and the morphological features of the cells can be efficiently preserved. The method used here allowed the visualization of several surface features of *C. neoformans*. It is a rapid and relatively simple technique that offers the opportunity to observe the architecture of *C. neoformans* cells.

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References

Casadevall, A., Perfect, J.R., 1998. Biochemistry. *Cryptococcus neoformans*. American Society for Microbiology, Washington, D.C., pp. 71–114.

Casali, A.K., Goulart, L., Rosa e Silva, L.K., Ribeiro, A.M., Amaral, A.A., Alves, S.H., et al., 2003. Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. FEMS Yeast Res. 3, 405–415.

Cleare, W., Casadevall, A., 1999. Scanning electron microscopy of encapsulated and non-encapsulated *Cryptococcus neoformans* and the effect of glucose on capsular polysaccharide release. Med. Mycol. 37, 235–243.

Franzot, S.P., Salkin, I.F., Casadevall, A., 1999. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. J. Clin. Microbiol. 37 (3), 838–840.

Janbon, G., 2004. *Cryptococcus neoformans* capsule biosynthesis and regulation. FEMS Yeast Res. 4, 765–771.

Horta, J.A., Staats, C.C., Casali, A.K., Ribeiro, Â.M., Schrank, I.S., Schrank, A., et al., 2002. Epidemiological aspects of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. Med. Mycol. 40, 1–7.

Katsu, M., Kidd, S., Ando, A., Moretti-Branchini, M.L., Mikami, Y., Nishimura, K., et al., 2004. The internal transcribed spacers and 5.8 S rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* species complex. FEMS Yeast Res. 4, 377–388.

Nosanchuk, J.D., Cleare, W., Franzot, S.P., Casadevall, A., 1999. Amphotericin B and Fluconazole affect cellular charge, macrophage phagocytosis, and cellular morphology of *Cryptococcus neoformans* at sub inhibitory concentrations. Antimicrob. Agents Chemother. 43 (2), 233–239.

Sorrell, T.C., 2001. *Cryptococcus neoformans* variety *gattii*. Med. Mycol. 39, 155–168.

5. CONCLUSÕES E PERSPECTIVAS

O método de RDA genômico mostrou-se útil para a identificação de seqüências polimórficas em genomas mais complexos (quando comparados aos genomas procarióticos). De todas as seqüências identificadas, já foram caracterizados os polimorfismos do gene putativo IDE em um estudo baseado na filogenia de múltiplos genes desenvolvido em parceria com Wieland Meyer (Molecular Mycology Research Laboratory, CIDM, University of Sydney, Austrália). A análise do *locus* IDE foi realizada como parte desta tese, enquanto a análise dos outros 3 *loci* estudados (ACT1, URA5 e PLB1) foi desenvolvida pelo grupo da Universidade de Sydney. Este estudo demonstrou que o conceito atualmente empregado de duas espécies para o complexo *C. neoformans* é válido e suporta os tipos moleculares previamente estabelecidos pelas análises de RFLP, M13 fingerprinting e AFLP.

Também foi desenvolvido um método alternativo para o processamento de amostras de *C. neoformans* e *C. gattii* para MEV. Foram confirmados dados de outros estudos sobre a morfologia celular das espécies do complexo *C. neoformans*, mostrando que a formação de cápsula polissacarídica em *C. gattii* é mais pronunciada e independe do meio de cultivo, quando comparado a *C. neoformans*. O método pode ser facilmente aplicado a outras espécies de leveduras ou no estudo da interação entre espécies, como já foi demonstrado por FUENTEFRIA *et al*. (2007).

Como perspectivas deste trabalho, temos o desenvolvimento de *primers* específicos e a padronização de reações de PCR para outras seqüências

identificadas pelo RDA. Posteriormente, poderá ser realizada a caracterização de isolados clínicos e ambientais do Brasil e de diversas regiões do mundo quanto à presença de polimorfismos utilizando PCR e seqüenciamento das regiões amplificadas. Este tipo de caracterização de isolados do complexo *C. neoformans* pode contribuir para o estudo da distribuição dos tipos moleculares deste patógeno, que vem mudando em nível mundial nos últimos anos.

6. COLABORAÇÕES

No decorrer deste trabalho, outros estudos relacionados à biologia de *C. neoformans* e identificação de espécies de leveduras foram desenvolvidos em parceria com colegas de Mestrado e Doutorado neste programa de pós-graduação.

ANEXO 1

LEAL, A. L.; FAGANELLO, J.; BASSANESI, M. C. & VAINSTEIN, M.H. *Cryptococcus* species identification by multiplex PCR. *Med Mycol*, *in press*, 2008.

ANEXO 2

FUENTEFRIA, A. M.; SUH, S-O.; LANDELL, M. F.; FAGANELLO, J.; SCHRANK, A.; VAINSTEIN, M. H.; BLACKWELL, M. & VALENTE, P. *Trichosporon insectorum* sp. nov., a new anamorphic basidiomycetous killer yeast. *Mycol Res*, 112:93-99, 2008.

ANEXO 3

FUENTEFRIA, A. M.; FAGANELLO, J.; PAZZINI, F.; SCHRANK, A.; VALENTE, P. & VAINSTEIN, M. Typing and patterns of cellular morphological alterations in *Cryptococcus neoformans* and *Cryptococcus gattii* isolates exposed to a panel of killer yeasts. *Med Mycol*, 45:503-512, 2007.

ANEXO 4

ABEGG, M. A.; CELLA, F. L.; FAGANELLO, J.; VALENTE, P.; SCHRANK, A. & VAINSTEIN, M. H. *Cryptococcus neoformans* and *Cryptococcus gattii* isolated from the excreta of Psittaciformes in a Southern Brazilian Zoological Garden. *Mycopathologia*, 161: 83–91, 2006.

ANEXO 5

HORTA, J. A.; FAGANELLO, J.; ROSA E SILVA, L. K.; OLIVEIRA, L. T.; SANTURIO, J. M.; VAINSTEIN, M. H. & ALVES, S. H. Susceptibility to heat and antifungal agents of *Cryptococcus neoformans* var. *neoformans* (serotype D) isolated from *Eucalyptus* spp. in Rio Grande do Sul, Brazil. *Braz J Microbiol*, 36:1-6, 2005.

7. REFERÊNCIAS BIBLIOGRÁFICAS

ABEGG, M. A.; CELLA, F. L.; FAGANELLO, J.; VALENTE, P.; SCHRANK, A. & VAINSTEIN, M. H. *Cryptococcus neoformans* and *Cryptococcus gattii* isolated from the excreta of Psittaciformes in a Southern Brazilian Zoological Garden. *Mycopathologia*, 161: 83–91, 2006.

ABEGG, M. A. Isolamento e caracterização de C.*neoformans* a partir de potenciais reservatórios ambientais inexplorados no Rio Grande do Sul. Dissertação de Mestrado, Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal do Rio Grande do Sul, 2003.

ALLEN, N. L.; HILTON, A. C.; BETTS, R. & PENN, C. W. Use of representational difference analysis to identify *Escherichia coli* 0157- specific DNA sequences. *FEMS Microbiol Lett*, 197: 195-201, 2001.

ALSPAUGH, J. A.; PERFECT, J. R. & HEITMAN, J. Signal transduction pathways regulating differentiation and pathogenicity of *Cryptococcus neoformans*. *Fungal Gen Biol*, 25: 1-14, 1998.

ALZATE, J. F.; RAMÍREZ-PINEDA, J. R.; GONZÁLEZ, V. M.; PATIÑO, E.; VÉLEZ, I. D. & JIMÉNEZ-RUIZ, A. *Leishmania* (*Viannia*) *panamensis*: Cloning of the histone H1 genes by representational difference analysis. *Exp Parasitol*, 112: 126–129, 2006.

BAEZA, L. C.; BAILÃO, A. M.; BORGES, C. L.; PEREIRA, M.; SOARES, C. M. A. & GIANNINI, M. J. S. M. cDNA representational difference analysis used in the

identification of genes expressed by *Trichophyton rubrum* during contact with keratin. *Microbes Infect*, 9: 1415-1421, 2007.

BAILÃO, A. M.; SCHRANK, A.; BORGES, C. L.; PARENTE, J. A.; DUTRA, V.; FELIPE, M. S.; FIÚZA, R. B.; PEREIRA, M. & DE ALMEIDA SOARES, C. M. The transcriptional profile of *Paracoccidioides brasiliensis* yeast cells is influenced by human plasma. *FEMS Immunol Med Microbiol*, 51: 43-57, 2007.

BART, A.; DANKERT, J. & VAN DER ENDE, A. Representational difference analysis of *Neisseria meningitidis* identifies sequences that are specific for the hyper-virulent lineage III clone. *FEMS Microbiol Lett*, 188: 111-114, 2000.

BART, A.; PANNEKOEK, Y.; DANKERT, J. & VAN DER ENDE, A. *NmeSI* restriction-modification system identified by representational difference analysis of a hypervirulent *Neisseria meningitidis* strain. *Infect Immun*, 69: 1816-1820, 2001.

BOEKHOUT, T.; THEELEN, B.; DIAZ, M.; FELL, J. W.; HOP, W. C. J.; ABELN, E. C. A.; DROMER, F. & MEYER, W. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology*, 147: 891-907, 2001.

BOVERS, M.; HAGEN, F.; KURAMAE, E. E.; DIAZ, M. R.; SPANJAARD, L.; DROMER, F.; HOOGVELD, H. L. & BOEKHOUT, T. Unique hybrids between the fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*. *FEMS Yeast Res*, 6: 599-607, 2006.

BOVERS, M.; HAGEN, F.; KURAMAE, E. E. & BOEKHOUT, T. Six monophyletic lineages identified within *Cryptococcus neoformans* and *Cryptococcus gattii* by multi-locus sequence typing. *Fungal Genet Biol*, 45: 400-421, 2008.

BRANDT, M. E.; BRAGG, S. L. & PINNER, R. W. Multilocus enzyme typing of *Cryptococcus neoformans*. *J Clin Microbiol*, 31: 2819-2823, 1993.

BUCHANAN, K. L. & MURPHY, J. W. What makes *Cryptococcus neoformans* a pathogen? *Emerg Infect Dis*, 4: 71-83, 1998.

CALIA, K. E.; WALDOR, M. K. & CALDERWOOD, S. B. Use of Representational Difference Analysis to identify genomic differences between pathogenic strains of *Vibrio cholerae*. *Infect Immun*, 66: 849-852, 1998.

CASADEVALL, A. & PERFECT, J. R. *Cryptococcus neoformans*. Washington, American Society for Microbiology, 1998.

CASALI, A. K.; GOULART, L.; SILVA, L. K. R.; RIBEIRO, A. M.; AMARAL, A. A.; ALVES, S. H.; SCHRANK, A.; MEYER, W. & VAINSTEIN, M. H. Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *FEMS Yeast Res*, 3: 405-415, 2003.

CHEN, F.; CURRIE, B. P.; CHEN, L-C.; SPITZER, S. G.; SPITZER, E. D. & CASADEVALL, A. Genetic relatedness of *Cryptococcus neoformans* clinical isolates grouped with the repetitive DNA probe CNRE-1. *J Clin Microbiol*, 33: 2818-2822, 1995.

CHEN, S. C. A.; BROWNLEE, A. G.; SORRELL, T. C.; RUMA, P.; ELLIS, D. H.; PFEIFFER, T.; SPEED, B. R. & NIMMO, G. Identification by Random Amplification of Polymorphic DNA of a common molecular type of *Cryptococcus neoformans* var. *neoformans* in patients with AIDS or other immunosuppressive conditions. *J Infect Dis*, 8: 173-754, 1996.

CHOI, J. Y.; SIFRI, C. D.; GOUMNEROV, B. C.; RAHRNE, L. G.; AUSUBEL, F. M. & CALDERWOOD, S. B. Identification of virulence genes in a pathogenic strain of *Pseudomonas aeruginosa* by Representational Difference Analysis. *J Bacteriol*, 184: 952-961, 2002.

CLEARE, W. & CASADEVALL, A. Scanning electron microscopy of encapsulated and non-encapsulated *Cryptococcus neoformans* and the effect of glucose on capsular polysaccharide release. *Med Mycol*, 37: 235-243, 1999.

COX, G.M.; MUKHERJEE, J.; COLE, G.T.; CASADEVALL, A. & PERFECT, J.R. Urease as a virulence factor in experimental cryptococcosis. *Infect Immun*, 68: 443–448, 2000.

COX, G. M.; MCDADE, H. C.; CHEN, S. C.; TUCKER, S. C.; GOTTFREDSSON, M.; WRIGHT, L. C.; SORRELL, T. C.; LEIDICH, S. D.; CASADEVALL, A.; GHANNOUM, M. A. & PERFECT, J. R. Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. *Mol Microbiol* , 39: 166–175, 2001.

DEL VALLE, L. & PIÑA-OVIEDO, S. HIV disorders of the brain: pathology and pathogenesis. *Front Biosci*, 11: 718-732, 2006.

DIAZ, M. R.; BOEKHOUT, T.; THEELEN, B. & FELL, J. W. Molecular sequence analyses of the intergenic spacer (IGS) associated with rDNA of the two varieties of the pathogenic yeast, *Cryptococcus neoformans*. *System Appl Microbiol*, 23: 535-545, 2000.

DREW, A. C. & BRINDLEY, P. J. Female-specific sequences isolated from *Schistossoma mansoni* by representational difference analysis. *Mol Biochem Parasitol*, 71: 173-181, 1995.

DROUHET, E. Milestones in the history of *Cryptococcus* and cryptococcosis. *J Mycol Méd*, 7: 10-27, 1997.

DUTRA, V.; NAKASATO, L.; BROETTO, L.; SCHRANK, I. S.; VAINSTEIN, M. H. & SCHRANK, A. Application of representational difference analysis to identify sequence tags expressed by *Metharizium anisopliae* during the infection process of the tick Boophilus microplus cuticle. *Res Microbiol*, 155: 245-251, 2004.

EDWARDS, M. R.; GORDON, M. A.; LAPA, E. W. & GHIORSE, W. C. Micromorphology of *Cryptococcus neoformans*. *J Bacteriol*, 94: 766-777, 1967.

EISENMAN, H. C.; MUES, M.; WEBER, S. E.; FRASES, S.; CHASKES, S.; GERFEN, G. & CASADEVALL, A. *Cryptococcus neoformans* laccase catalyses melanin synthesis from both D- and L-DOPA. *Microbiology*, 153:3954-3962, 2007.

FAN, M.; CURRIE, B. P.; GUTELL, R. R.; RAGAN, M. A. & CASADEVALL, A. The 16S-like, 5,8S and 23S-like rRNAs of the two varieties of *Cryptococcus neoformans*:

sequence, secondary structure, phylogenetic analysis and restriction fragment polymorphisms. *J Med Vet Mycol*, 32: 163-180, 1994.

FRASER, J. A.; GILES, S. S.; WENINK, E. C.; GEUNES-BOYER S. G.; WRIGHT, J. R.; DIEZMANN, S.; ALLEN, A.; STAJICH, J. E.; DIETRICH, F. S.; PERFECT, J. R. & HEITMAN, J. Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature*, 437:1360-1364, 2005.

FRANZOT, S. P.; HAMDAN, J. S.; CURRIE, B. P. & CASADEVALL, A. Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: evidence for both local genetic differences and a global clonal population structure. *J Clin Microbiol*, 35: 2243-2251, 1997.

FRANZOT, S. P.; SALKIN, I. F. & CASADEVALL, A. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. *J Clin Microbiol*, 37: 838-840, 1999.

FUENTEFRIA, A. M.; FAGANELLO, J.; PAZZINI, F.; SCHRANK, A.; VALENTE, P. & VAINSTEIN M. Typing and patterns of cellular morphological alterations in *Cryptococcus neoformans* and *Cryptococcus gattii* isolates exposed to a panel of killer yeasts. *Med Mycol*, 45: 503-12, 2007.

HALLIDAY, C. L. & CARTER, D. A. Clonal reproduction and limited dispersal in na environmental population of *Cryptococcus neoformans* var. *gattii* isolates from Australia. *J Clin Microbiol*, 41: 703-711, 2003.

HEITMAN, J. Sexual reproduction and the evolution of microbial pathogens. *Curr Biol*, 16: 711-725, 2006.

HOANG, L. M.; MAGUIRE, J. A.; DOYLE, P.; FYFE, M. & ROSCOE, D. L. *Cryptococcus neoformans* infections at Vancouver Hospital and Health Sciences Centre (1997–-2002): epidemiology, microbiology and histopathology. *J Med Microbiol*, 53: 935–-940, 2004.

HORTA, J. A.; STAATS, C. C.; CASALI, A. K.; RIBEIRO, Â. M.; SCHRANK, I. S.; SCHRANK, A. & VAINSTEIN, M. H. Epidemiological aspects of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *Med Mycol*, 40: 1-7, 2002.

HUBANK, M. & SCHATZ, D. G. Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res*, 22: 5640-5648, 1994.

HULL, C. M. & HEITMAN, J. Genetics of *Cryptococcus neoformans*. *Annu Rev Genet*, 36: 557-615, 2002.

IWOBI, A.; RAKIN, A.; GARCIA, E. & HEESEMANN, J. Representational difference analysis uncovers a novel IS10-like insertion element unique to pathogenic strains of Yersinia enterocolitica. *FEMS Microbiol Lett*, 210: 251-255, 2002.

JAIN, N.; GUERRERO, A. & FRIES, B. C. Phenotypic switching and its implications for the pathogenesis of *Cryptococcus neoformans*. *FEMS Yeast Res*, 6:480-488, 2006.

KATSU, M.; KIDD, S.; ANDO, A.; MORETTI-BRANCHINI, M. L.; MIKAMI, Y.; NISHIMURA, K. & MEYER, W. The internal transcribed spacers and 5.8S rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* species complex. *FEMS Yeast Res*, 4: 377-388, 2004.

KHAWCHAROENPORN, T.; APISARNTHANARAK, A. & MUNDY, L. M. Non-*neoformans* cryptococcal infections: a systematic review. *Infection*, 35: 51-8, 2007.

KIDD, S. E.; CHOW, Y.; MAK, S.; BACH, P. J.; CHEN, H.; HINGSTON, A. O.; KRONSTAD, J. W. & BARTLETT, K. H. Characterization of environmental sources of the human and animal pathogen *Cryptococcus gattii* in British Columbia, Canada, and the Pacific Northwest of the United States. *Appl Environ Microbiol*, 73: 1433-1443, 2007.

KWON-CHUNG, K. J.; EDMAN, J. C. & WICKES, B. L. Genetic association of *mating type*s and virulence in *Cryptococcus neoformans*. *Infect Immun*, 60: 602–605, 1992.

KWON-CHUNG, K. J. & BENNETT, J. E. Distribution of α and a *mating type*s of *Cryptococcus neoformans* among natural and clinical isolates. *Am J Epidem*, 108: 337 – 340, 1978.

KWON-CHUNG, K. J. & VARMA, A. Do major species concepts support one, two or more species within *Cryptococcus neoformans*? *FEMS Yeast Res*, 6: 574-587, 2006.

LENGELER, K. B.; COX, G. M. & HEITMAN, J. Serotype AD Strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus *Infect Immun*, 69: 115–122, 2001.

LIN, X.; HULL, C. M. & HEITMAN, J. Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans*. *Nature*, 434: 1017-1021, 2005.

LIN, X.; LITVINTSEVA, A. P.; NIELSEN, K.; PATEL, S.; FLOYD, A.; MITCHELL, T. G. & HEITMAN, J. Alpha AD alpha hybrids of *Cryptococcus neoformans*: evidence of same-sex mating in nature and hybrid fitness. *PLoS Genet*, 3:1975-1990, 2007.

LIN, X. & HEITMAN, J. The biology of the *Cryptococcus neoformans* species complex. *Annu Rev Microbiol*, 60: 69-105, 2006.

LISITSYN, N. A.; LISITSYN, N. & WIGLER, M. Cloning the differences between two complex genomes. *Science*, 259: 946-951, 1993.

LISITSYN, N. A. Representational difference analysis: finding the differences between genomes. *TIG*, 11: 303-307, 1995.

LITVINTSEVA, A. P.; THAKUR, R.; VILGALYS, R. & MITCHELL, T. G. Multilocus Sequence Typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (serotype A), including a unique population in Botswana. *Genetics*, 172: 2223–2238, 2006.

LOFTUS, B. J.; FUNG, E.; RONCAGLIA, P.; ROWLEY, D.; AMEDEO, P.; BRUNO, D.; VAMATHEVAN, J.; MIRANDA, M.; ANDERSON, I. J.; FRASER, J. A.; ALLEN, J. E.;

BOSDET, I. E.; BRENT, M. R.; CHIU, R.; DOERING, T. L.; DONLIN, M. J.; D'SOUZA, C. A.; FOX, D. S.; GRINBERG, V.; FU, J.; FUKUSHIMA, M.; HAAS, B. J.; HUANG, J. C.; JANBON, G.; JONES, S. J.; KOO, H. L.; KRZYWINSKI, M. I.; KWON-CHUNG, J. K.; LENGELER, K. B.; MAITI, R.; MARRA, M. A.; MARRA, R. E.; MATHEWSON, C. A.; MITCHELL, T. G.; PERTEA, M.; RIGGS, F. R.; SALZBERG, S. L.; SCHEIN, J. E.; SHVARTSBEYN, A.; SHIN, H.; SHUMWAY, M.; SPECHT, C. A.; SUH, B. B.; TENNEY, A.; UTTERBACK, T. R.; WICKES, B. L.; WORTMAN, J. R.; WYE, N. H.; KRONSTAD, J. W.; LODGE, J. K.; HEITMAN, J.; DAVIS, R. W.; FRASER, C. M. & HYMAN, R. W. The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. *Science*, 307: 1321–1324, 2005.

MARTINEZ, L. R. & CASADEVALL, A. *Cryptococcus neoformans* biofilm formation depends on surface support and carbon source and reduces fungal cell susceptibility to heat, cold, and UV light. *Appl Environ Microbiol*, 73: 4592–4601, 2007.

McCLELLAND, C. M.; CHANG, Y. C.; VARMA, A. & KWON-CHUNG, K. J. Uniqueness of the mating system in *Cryptococcus neoformans*. *Trends Microbiol*, 12: 208-212, 2004.

McCLELLAND, C. M.; CHANG, Y. C. & KWON-CHUNG, K. J. High frequency transformation of Cryptococcus neoformans and Cryptococcus gattii by Agrobacterium tumefaciens. *Fungal Genet Biol*, 42 :904-913, 2005.

McFADDEN, D.; ZARAGOZA, O. & CASADEVALL, A. The capsular dynamics of *Cryptococcus neoformans*. *Trends Microbiol*, 14: 497-505, 2006.

MEDEIROS RIBEIRO, A.; SILVA, L. K.; SILVEIRA SCHRANK, I.; SCHRANK, A; MEYER, W. & HENNING VAINSTEIN, M. Isolation of *Cryptococcus neoformans* var. *neoformans* serotype D from Eucalypts in South Brazil. *Med Mycol*, 44:707-13, 2006.

MEYER, W.; MARSZEWSKA, K.; AMIRMOSTOFIAN, M.; IGREJA, R. P.; HARDTKE, C.; METHLING, K.; VIVIANI, M. A.; CHINDAMPORN, A.; SUKROONGREUNG, S.; JOHN, M. A.; ELLIS, D. H. & SORRELL, T. C. 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*, 20:1790-1799, 1999.

Ministério da Saúde. Dados e pesquisa em DST e AIDS. Brasília, Coordenação do Programa Nacional de DST/AIDS, 2002.

MITCHELL, T. G. & PERFECT, J. R. Cryptococcosis in the Era of AIDS – 100 years after the discovery of *Cryptococcus neoformans*. *Clin Microbiol Rev*, 8: 515-548, 1995.

MIYAZAKI, Y.; NAKAMURA, M. & BABASAKI, K. Molecular cloning of developmentally specific genes by representational diVerence analysis during the fruiting body formation in the basidiomycete *Lentinula edodes*. *Fungal Genet Biol*, 42: 493–505, 2005.

NISHIKAWA, M. M.; LAZERA, M. S.; BARBOSA, G. G.; TRILLES, L.; BALASSIANO, B. R.; MACEDO, R. C.; BEZERRA, C. C. F.; PÉREZ, M. A.; CARDARELLI, P. & WANKE, B. Serotyping of 467 *Cryptococcus neoformans* isolates from clinical and

environmental sources in Brazil: Analysis of host and regional patterns. *J Clin Microbiol*, 41: 73-77, 2003.

NOSANCHUK, J. D.; CLEARE, W.; FRANZOT, S. P. & CASADEVALL, A. Amphotericin B and Fluconazole affect cellular charge, macrophage phagocytosis, and cellular morphology of *Cryptococcus neoformans* at subinhibitory concentrations. *Antimicrob Ag Chemother*, 43: 233-239, 1999.

NOSANCHUK, J. D. & CASADEVALL, A. Budding of melanized *Cryptococcus neoformans* in the presence or absence of L-dopa. *Microbiology*, 149: 1945-1951, 2003.

PAPPALARDO, M. C. S. M. & MELHEM, M. S. C. Cryptococcosis: A review of the Brazilian experience for the disease. *Rev Inst Med Trop S Paulo*, 45: 299-305, 2003.

PASTORIAN, K.; HAWELL, L. & BYUS, C. V. Optimization of cDNA Representational Difference Analysis for the identification of differentially expressed mRNAs. *Anal Biochem*, 283: 89-98, 2000.

PERFECT, J. R. *Cryptococcus neoformans*: a sugar-coated killer with designer genes. *FEMS Immunol Med Microbiol*, 45:395–404, 2005.

PERFECT, J. R. *Cryptococcus neoformans*: the yeast that likes it hot. *FEMS Yeast Res*, 6: 463–468, 2006.

ROSA E SILVA, L. K.; STAATS, C. C.; GOULART, L. S.; MORELLO, L. G.; PELEGRINELLI FUNGARO, M. H.; SCHRANK, A. & VAINSTEIN, M. H.

Identification of novel temperature-regulated genes in the human pathogen *Cryptococcus neoformans* using representational difference analysis. *Res Microbiol*, 2008, doi:10.1016/j.resmic.2007.12.006.

ROTHKAMP, A.; STROMMENGER, B. & GERLACH, G-F. Identification of *Brachyspira hyodysenteriae*-specific DNA fragments using representational difference analysis. *FEMS Microbiol Letters*, 210: 173-179, 2002.

ROY, S. W.; PENNY, D. & NEAFSEY, D. E. Evolutionary conservation of UTR intron boundaries in *Cryptococcus*. *Mol Biol Evol*, 24:1140-1148, 2007.

SCHEIN, J. E.; TANGEN, K. L.; CHIU, R.; SHIN, H.; LENGELER, K. B.; MACDONALD, W. K.; BOSDET, I.; HEITMAN, J.; JONES, S. J. M.; MARRA, M. A. & KRONSTAD, J. W. Physical maps for genome analysis of serotype A and D strains of the fungal pathogen *Cryptococcus neoformans*. *Genome Res*, 12: 1445-1453, 2002.

SHARPTON, T. J.; NEAFSEY, D. E.; GALAGAN, J. E. & TAYLOR, J. W. Mechanisms of intron gain and loss in *Cryptococcus*. *Genome Biol*, 9:R24, 2008.

SIDRIM, J. J. C. & MOREIRA, J. L. B. Fundamentos clínicos e laboratoriais da micologia médica. 1ª ed., Guanabara Koogan, 1999.

SORRELL, T. C.; CHEN, S. C. A.; RUMA, P.; MEYER, W.; PFEIFFER T. J.; ELLIS, D. H. & BROWNLEE, A. G. Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. gattii by Random Amplification of Polymorphic DNA analysis and PCR fingerprinting. *J Clin Microbiol*, 34: 1253-1260, 1996.

SPITZER, S. G. & SPITZER, E. D. Isolation of *Cryptococcus neoformans* chromosome-specific probes using expressed sequence tags. *J Med Vet Mycol*, 35: 257-261, 1997.

STEENBERGEN, J. N. & CASADEVALL, A. The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. *Microbes Infect*, 5: 667-675, 2003.

STEINBACH, W. J.; REEDY, J. L.; CRAMER, R. A. JR.; PERFECT, J. R. & HEITMAN, J. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. *Nat Rev Microbiol*, 5: 418-430, 2007.

SUKROONGREUNG, S.; KITINIYOM, K.; NILAKUL, C. & TANTIMAVANICH, S. Pathogenicity of basidiospores of Filobasidiela *neoformans* var. *neoformans*. *Med Mycol*, 36: 419-424, 1998.

TSCHARKE, R. L.; LAZERA, M.; CHANG, Y. C.; WICKES, B. L. & KWON-CHUNG, K. J. Haploid fruiting in *Cryptococcus neoformans* is not *mating type* α-specific. *Fungal Genet Biol*, 39: 230-237, 2003.

UPTON, A.; FRASER, J. A.; KIDD, S. E.; BRETZ, C.; BARTLETT, K. H.; HEITMAN, J. & MARR, K. A. First Contemporary Case of Human Infection with *Cryptococcus gattii* in Puget Sound: Evidence for Spread of the Vancouver Island Outbreak. *J Clin Microbiol*, 45: 3086-3088, 2007.

WANG, Y.; AISEN, P. & CASADEVALL, A. Melanin, melanin “ghosts”, and melanin composition in *Cryptococcus neoformans*. *Infect Immun*, 64: 2420-2424, 1996.

WICKES, B. L.; MAYORGA, M. E.; EDMAN, U. & EDMAN, J. C. Dimorphism and haploid fruiting in *Cryptococcus neoformans*: Association with the *mating type*. *Proc Natl Acad Sci USA*, 93: 7327-7331, 1996.

XU, J.; VILGALYS, R. & MITCHELL, T. G. Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. *Mol Ecol*, 9: 1471-1481, 2000.

XU, J. & MITCHELL, T. G. Comparative gene genealogical analyses of strains of serotype AD identify recombination in populations of serotypes A and D in the human pathogenic yeast *Cryptococcus neoformans*. *Microbiology*, 149: 2147-2154, 2003.

ZERPA, R.; HUICHO, L. & GUILLÉN, A. Modified India ink preparation for *Cryptococcus neoformans* in cerebrospinal fluid specimens. *J Clin Microbiol*, 34: 2290-2291, 1996.

8. CURRICULUM VITAE (RESUMIDO)

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Produção em C, T & A

Produção bibliográfica

Artigos completos publicados em periódicos

1. LEAL, A. L., FAGANELLO, J., FUENTEFRIA, A. M., BOLDO, J. T., BASSANESI, M. C., VAINSTEIN, M. H.
   Epidemiological profile of cyptococcal meningitis patients in Rio Grande do Sul, Brazil. Mycopathologia, 2008.
2. LEAL, A. L., FAGANELLO, J., BASSANESI, M. C., VAINSTEIN, M. H.
   Cryptococcus species identification by multiplex PCR.. Medical Mycology (Oxford), 2008.

3. FUENTEFRIA, A. M., SUH, S., LANDELL, M. F., FAGANELLO, J., SCHRANK, A., VAINSTEIN, M. H., BLACKWELL, M., VALENTE, P.
   Trichosporon insectorum sp. nov., a new anamorphic basidiomycetous killer yeast.. Mycological Research. , v.112, p.93 - 99, 2008.
4. FUENTEFRIA, A. M., FAGANELLO, J., PAZZINI, F., SCHRANK, A., VALENTE, P., VAINSTEIN, M. H.
   Typing and patterns of cellular morphological alterations in Cryptococcus neoformans and Cryptococcus gattii isolates exposed to a panel of killer yeasts.. Medical Mycology (Oxford). , v.45, p.503 - 512, 2007.
5. FAGANELLO, J., ARRUDA, W., SCHRANK, A., VAINSTEIN, M. H.
   An alternative method to prepare samples of the pathogenic yeast Cryptococcus neoformans for scanning electron microscopy analysis. Journal of Microbiological Methods. , v.64, p.416 - 419, 2006.
6. ABEGG, M. A., CELLA, F. L., FAGANELLO, J., VALENTE, P., SCHRANK, A., VAINSTEIN, M. H.
   Cryptococcus neoformans and Cryptococcus gattii Isolated from the Excreta of Psittaciformes in a Southern Brazilian Zoological Garden. Mycopathologia. , v.161, p.83 - 91, 2006.
7. HORTA, J., FAGANELLO, J., SILVA, L. K. R. E., SANTURIO, J. M., VAINSTEIN, M. H., ALVES, S. H.
   Susceptibility to heat and antifungal agents of Cryptococcus neoformans var. neoformans (serotype D) from Eucalyptus spp. in Rio Grande do Sul, Brazil. Brazilian Journal of Microbiology. , v.36, p.1 - 6, 2005.

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2. **XXIII Congresso Brasileiro de Microbiologia**, 2005. (Congresso)
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4. **XXIV Reunião de Genética de Microrganismos**, 2004. (Congresso)
5. **XXII Congresso Brasileiro de Microbiologia**, 2003. (Congresso)
6. **I Encontro Gaúcho de Genética, Biologia Molecular e Saúde**, 2003. (Encontro)
7. **Método Científico & Fronteiras do Conhecimento**, 2002. (Seminário)
8. **Jornada Farmacêutica Prof. Zósymo Lopes dos Santos**, 2001. (Outra)
9. **XV Congresso Brasileiro de Análises Clínicas / XXVIII Congresso Latinoamericano de Bioquímica Clínica**, 2001. (Congresso)
10. **8o Semana Acadêmica de Farmácia e Bioquímica**, 2000. (Outra)
11. **7o Semana Acadêmica de Farmácia e Bioquímica**, 1999. (Outra)
12. **10o Encontro Nacional de Química Analítica**, 1999. (Encontro)
13. **I Jornada de Atualização em Análises Clínicas**, 1998. (Outra)

9. ANEXOS

ANEXO 1

LEAL, A. L.; FAGANELLO, J.; BASSANESI, M. C. & VAINSTEIN, M.H.

***Cryptococcus* species identification by multiplex PCR.**

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Short Communication

Cryptococcus species identification by multiplex PCR

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Members of the *Cryptococcus* species complex are encapsulated basidiomycetous yeasts, which can affect the central nervous system (CNS) and if untreated may cause meningitis. *Cryptococcus neoformans* is an opportunistic pathogen causing infections mainly in immunocompromised individuals. *Cryptococcus gattii* is a primary pathogen responsible for a high incidence of cryptococcomas in the lung and brain and shows a delayed response to antifungal therapy. The differentiation between the two species is primarily based on their growth on and color change of canavanine-glycine-bromothymol blue agar (CGB). Since this test is not always reliable, a multiplex PCR to identify both *Cryptococcus* species using more than 130 samples was standardized and the results obtained compared to those with the CGB test, using the Crypto Check serotyping kit as the standard. The multiplex PCR was shown to be more specific than the CGB test, in that results obtained with it were in agreement with those from serotyping all the samples, while the data from the CGB test disagreed with 6 out of 131 samples.

Keywords *Cryptococcus neoformans*, *Cryptococcus gattii*, multiplex PCR, CGB

Introduction

Cryptococcus neoformans and *Cryptococcus gattii* are pathogenic yeasts that infect both immunocompromised and healthy individuals. *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) are cosmopolitan, while *C. gattii*, recently raised to species status, is considered to be restricted to tropical and subtropical regions [1,2]. However, more recently, *C. gattii* has been isolated from humans, many different animal species and several environmental locations on Vancouver Island and its surrounding areas of the Canadian and USA mainland [3–6]. Differences between the diseases caused by these two species have been reported, such as the delayed response of *C. gattii* to antifungal therapy and a high frequency of cryptococcomas in the lung and the brain caused by this specie [7]. Moreover, *C. gattii* has been shown to be less susceptible to antifungal agents than *C. neoformans* [8]. These characteristics make it important for clinicians to obtain a fast and reliable identification of the two *Cryptococcus* species. The canavanine-glycine-bromothymol blue agar test (CGB) was proposed by Kwon-Chung *et al.* [9] to distinguish *C. neoformans* from *C. gattii* and since its introduction has been used in many laboratories [10–12]. However, some results suggest that a CGB-positive reaction alone is not enough to reliably discriminate the two species [13,14]. In this report we propose a multiplex PCR method to differentiate the species based on primers constructed earlier [15]. The method was tested on141 samples isolated in Brazil and 19 recovered from several countries around the world. The Crypto Check serotyping kit (Iatron, Tokyo, Japan) was used as standard to compare the PCR and the CGB results. The multiplex PCR presented in this work appears to be more specific than the CGB agar method.

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Materials and methods

Strains

All strains used in this study are listed in Table 1 and include 131 isolates recovered and previously identified by subculturing on CGB media (LACEN-Laboratório Central do Estado do Rio Grande do Sul, Porto Alegre, RS, Brazil) as *C. neoformans* (121 strains) and as *C. gattii* (10 isolates) [9]. As the number of *C. gattii* isolates was much smaller than *C. neoformans*, we also used 10 strains which had been described in previous reports from our laboratory [10,16] and an additional 15 global strains from Canada, Mexico, Australia, Thailand, New Zealand, USA, Paraguay, South Africa, India, Columbia and Greece (kindly provided by Dr Wieland Meyer) [5,17–19]. The isolates were serotyped by the Crypto Check kit (Iatron, Tokyo, Japan) according to the manufacturer's instructions [20].

DNA extraction

DNA was extracted as previously described [21].

Multiplex PCR

PCR reactions were carried out in a volume of 25 µl, containing; 10–20 ng of DNA, reaction buffer (10 mM Tris HCl pH 8.3; 50 mM KCl; 2.3 mm $MgCl_2$), 200 µM of deoxynucleotide triphosphates, 25 pmol of each primer and 1 U Taq DNA polymerase (CENBIOT Enzimas, Porto Alegre, Brazil). Primers were CNa-70S (5′- ATTGCGTCCACCAAGGAGCTC -3′) and CNa-70A (5′- ATTGCGTCCATGTTACGTGGC -3′) for *C. neoformans*; and CNb-49S (5′- ATTGCGTCCAAGGTGTTGTTG -3′) and CNb-49A (5′- ATTGCGTCCATCCAACCGTTATC -3′) for *C. gattii* [15]. An initial denaturation at 94°C for 8 min was followed by 35 1 min cycles at 94°C, annealing at 65°C for 1 min, elongation at 72°C for 2 min, and a final elongation at 72°C for 8 min. The PCR amplicons were electrophoresed on 1% agarose gels in 1X Tris-borate-EDTA (TBE) buffer at 100 V for 40 min and then stained in a solution of 0.5 µg/ml ethidium bromide.

Sensitivity and specificity tests

To evaluate the sensitivity of the method, dilutions of a control DNA (HC5 – serotype A, previously characterized [16]) were performed and subjected to the multiplex PCR. To test the primer specificity, the DNA from *Mycobacterium tuberculosis*, *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, *Sporothrix schenckii*, *Rhodotorula mucilaginosa*, *Cryptococcus luteolus*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Escherichia coli* and human DNA were subjected to multiplex PCR. The amplicons obtained for *C. neoformans* and *C. gattii* were compared with the sequences from the microorganisms listed above and with the human sequence using the GenBank database and the program BLASTN, to verify their specificity.

RFLP analysis

To confirm the species-specificity of the obtained PCR products six *C. gattii* and six *C. neoformans* amplicons were digested with the restriction endonuclease *Eco*RI (Invitrogen) according to the manufacturer's instructions.

Results

Multiplex PCR

The primer sequences were submitted to a BLASTN search in GenBank against the *C. neoformans* JEC21 complete genome. The sequence retrieved from the search flanked by the CNa-70S and CNa-70A primers corresponded to a region on chromosome 3 that includes the coding sequence of a putative aminotransferase gene. The sequence retrieved from the blast search flanked by the CNb-49S and CNb-49A primers corresponded to a region on chromosome 2, which includes the coding sequence of a putative polymerase gene. The multiplex PCR amplified DNA fragments of 695 and 448 bp for *C. neoformans* and *C. gattii*, respectively. According to the size of the fragments, 6 of the 131 isolates from LACEN were identified as *C. gattii* (4.58%) and remaining125 were found to be *C. neoformans* (95.42%) (Fig. 1). All PCR results were in accordance with the results obtained by serotyping. Comparing the serotype data with results with the CGB agar test, we found the latter gave 5 false positives and 1 false negative. The multiplex PCR method also confirmed the previous identification of the 24 *C. gattii* strains from our previous work and those provided as global isolates (see Table 1).

Sensitivity and specificity tests

Among the DNA dilutions tested we found the minimal amount of DNA which resulted in an amplification product with the proposed multiplex PCR was 1.25 ng (data not shown). None of the tested DNAs, from other microorganisms or humans, resulted in an amplification product using the multiplex PCR (Fig. 2 A, B). This was confirmed by blasting the obtained PCR products for *C. neoformans* and *C. gattii*

Table 1 Isolates used in this work.

Strain number	Origin	CGB#	Multiplex PCR#	Serotype*/Molecular Type	Species
L001	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L002	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L003	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L004	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L005	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L006	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L007	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L008	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L009	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L010	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L011	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L012	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L013	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L014	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
L015	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L016	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L017	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L018	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L019	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L020	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L021	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L022	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L023	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L024	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L025	**Brazil, Clinical**	***Cg***	***Cn***	A/VNI	***Cn* var *grubii***
L026	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L027	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L028	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L029	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
L030	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L031	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L032	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L033	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L034	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L035	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
L036	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L037	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L038	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L039	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L040	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L041	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L042	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L043	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L044	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L045	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L046	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L047	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L048	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L049	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L050	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L051	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L052	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L053	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L054	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L055	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L056	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L057	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L058	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
L059	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L060	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*

Table 1 (*Continued*)

Strain number	Origin	CGB#	Multiplex PCR#	Serotype*/Molecular Type	Species
L061	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L062	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L063	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L064	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L065	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L066	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L067	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L068	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L069	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L070	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L071	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L072	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L073	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L074	**Brazil, Clinical**	***Cg***	***Cn***	**A/VNI**	***Cn* var *grubii***
L075	**Brazil, Clinical**	***Cg***	***Cn***	**A/VNI**	***Cn* var *grubii***
L076	**Brazil, Clinical**	***Cg***	***Cn***	**A/VNI**	***Cn* var *grubii***
L077	**Brazil, Clinical**	***Cg***	***Cn***	**A/VNI**	***Cn* var *grubii***
L078	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L079	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L080	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L081	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L082	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L083	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L084	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L085	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L086	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L087	**Brazil, Clinical**	***Cn***	***Cg***	**B/VGIII**	***C. gattii***
L088	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L089	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L090	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L091	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L092	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L093	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L094	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L095	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L096	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L097	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L098	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L099	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L100	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L101	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L102	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L103	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L104	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L105	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L106	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L107	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L108	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L109	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L110	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L111	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L112	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L113	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L114	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L115	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L116	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L117	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L118	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L119	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L120	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*

Table 1 (*Continued*)

Strain number	Origin	CGB#	Multiplex PCR#	Serotype*/Molecular Type	Species
L121	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L122	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L123	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L124	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L125	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L126	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L127	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
L128	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L129	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L130	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
L131	Brazil, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
AL6	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
AL10	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
AL13	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
AL14	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
AL32	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
AL33	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
AL34	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
HSL3	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
C5	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
C43	Brazil, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
WM179	Australia, Clinical	*Cg*	*Cg*	B/VGI	*C. gattii*
WM178	Australia, Clinical	*Cg*	*Cg*	B/VGII	*C. gattii*
WM175	Australia, Clinical	*Cg*	*Cg*	B/VGIII	*C. gattii*
WM779	South Africa, Veterinary	*Cg*	*Cg*	C/VGIV	*C. gattii*
WM148	Australia, Clinical	*Cn*	*Cn*	A/VNI	*Cn* var *grubii*
WM626	Australia, Clinical	*Cn*	*Cn*	A/VNII	*Cn* var *grubii*
WM628	Australia, Clinical	*Cn*	*Cn*	AD/VNIII	*Cn* hybrid AD
ATCC28957		*Cn*	*Cn*	D/VNIV	*Cn* var *neoformans*
LA1	Mexico, Clinical	*Cg*	*Cg*	B/VGI	*C. gattii*
AV55	Greece, Clinical	*Cg*	*Cg*	B/VGII	*C. gattii*
MC-S-239	Thailand, Clinical	*Cg*	*Cg*	B/VGII	*C. gattii*
CN043	New Zealand, Clinical	*Cg*	*Cg*	C/VGIII	*C. gattii*
WM728	USA, Environmental	*Cg*	*Cg*	B/VGIII	*C. gattii*
LA290	Paraguay, Clinical	*Cg*	*Cg*	-/VGIII	*C. gattii*
B5748	India, Clinical	*Cg*	*Cg*	B/VGIV	*C. gattii*
LA568	Columbia, Clinical	*Cg*	*Cg*	B/VGIV	*C. gattii*
LA390	Mexico, Clinical	*Cg*	*Cg*	-/VGIV	*C. gattii*
R265	Canada, Clinical	*Cg*	*Cg*	B/VGII	*C. gattii*
R272	Canada, Clinical	*Cg*	*Cg*	B/VGII	*C. gattii*

Cn = *C. neoformans*; *Cg* = *C. gattii*.
Samples in bold indicates disagreements in identification among the tests used.
*Crypto Check serotyping kit (Iatron Labs, Tokyo, Japan).

against GenBank. According to *in silico* analysis, no significant identity among the amplicons from *C. neoformans* or *C. gattii* and sequences from other microorganisms or humans was found.

RFLP analysis

Digestion with the restriction endonuclease *Eco*RI resulted in fragments of 447 and 248 bp for *C. neoformans* and 324 and 124 bp for *C. gattii* (Fig. 3). These results confirmed the specificity of the amplified fragments for the respective species.

Discussion

The CGB test has become a traditional method to differentiate *C. neoformans* from *C. gattii* ever since it was proposed in 1982 [9,10,22]. One problem with this test is the possibility of erroneous results, creating non-reliable diagnostic data in laboratories where no other identification methods are available. Moreover, false positive reactions have been reported, suggesting that CGB alone is not sufficient to accurately discriminate between the two species [13,14]. The results of the CGB test in the current work were found to be 95.4% in agreement (125 out of 131 samples) with the serotyping

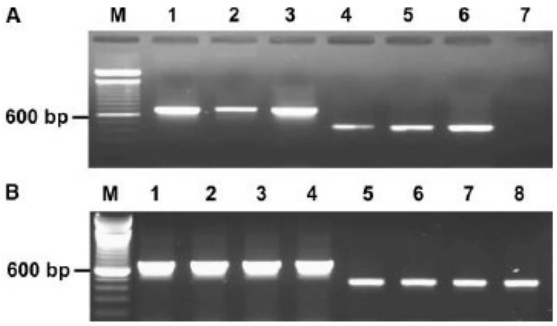

Fig. 1 *Cryptococcus* species identification by multiplex PCR. Agarose gel electrophoresis of products amplified by the multiplex PCR using the primer pairs CNA70A – CNA70S and CNB49A – CNB49S. (A) Lanes 1 to 3, *Cryptococcus neoformans* var. *grubii* and lanes 4 to 6, *Cryptococcus gattii*; lane 7, negative control. (B) Amplification of the major molecular types of this species complex as identified by *URA5*-RFLP and PCR-fingerprinting [23]. Lanes 1 to 8, WM148 (VNI, serotype A), WM626 (VNII, serotype A), WM628 (VNIII, serotype AD) and ATCC28957 (VNIV, serotype D), WM179 (VGI, serotype B), WM178 (VGII, serotype B), WM175 (VGIII, serotype B) and WM779 (VGIV, serotype C). The DNA marker (100bp DNA ladder, Invitrogen) is indicated on the left side (M).

data. Five were false positive and one false negative were noted, confirming the need for other procedures to obtain a trustworthy identification.

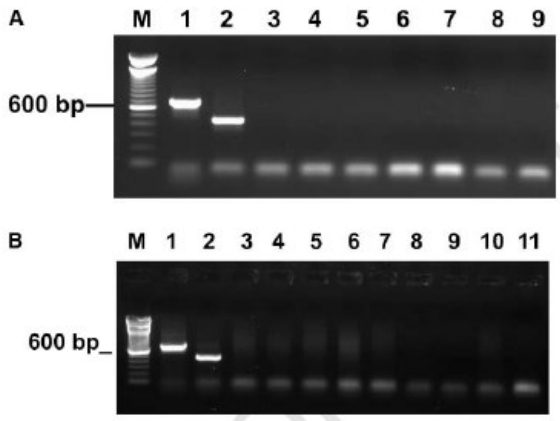

Fig. 2 Specificity test of the multiplex PCR. Agarose gel electrophoresis of products amplified by the multiplex PCR using the primer pairs CNA70A – CNA70S and CNB49A – CNB49S from different microorganisms and human. *Cryptococcus neoformans* var. *grubii* (lane 1) and *Cryptococcus gattii* (lane 2) were used as internal control. (A) Lanes (3) *S. aureus*, (4) *M. tuberculosis*, (5) *C. albicans*, (6) *E. coli*, (7) *N. meningitidis*, (8) human DNA, (9) negative control. (B) Lanes (3) *C. dubliniensis*, (4) *C. parapsilosis*, (5) *C. tropicalis*, (6) *C. guilliermondii*, (7) *C. krusei*, (8) *S. schenckii*, (9) *R. mucilaginosa*, (10) *C. luteolus*, (11) negative control. The DNA marker (100 bp DNA ladder, Invitrogen) is indicated on the left side (M).

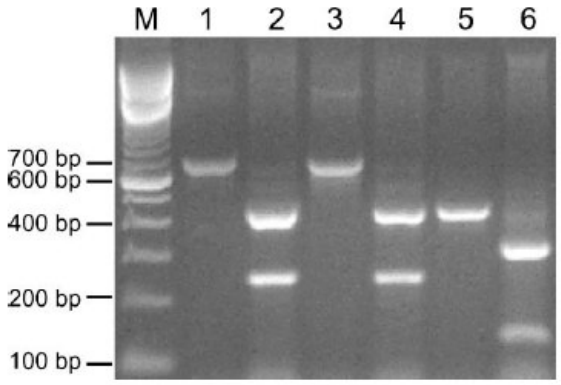

Fig. 3 RFLP analysis of multiplex PCR products. Agarose gel electrophoresis of products digested with the restriction endonuclease *Eco*RI. Lanes 1, 3 and 5, *Cryptococcus neoformans* var. *grubii*, *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus gattii* PCR products, respectively; lanes 2, 4 and 6, *Eco*RI digested *Cryptococcus neoformans* var. *grubii*, *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus gattii* PCR products, respectively. The DNA marker (100 bp DNA ladder, Invitrogen) is indicated on the left side (M).

The proposed multiplex PCR is a simple, fast and species-specific test, which did not amplify DNA from other microorganisms or humans, and showed potential applicability for correctly identifying *Cryptococcus* species within the tested samples. Serotyping using the Crypto Check kit was used as a gold standard in this study. The multiplex PCR identifications were in 100% agreement with those obtained through serotyping. The PCR was more accurate than the CGB test in six (4.58%) of the samples, proving it to be an efficient method. Since the Crypto Check kit is no longer produced and no similar kits are commercially available there is a rising need for the development of new methods for use in clinical diagnostic laboratory to identify the two *Cryptococcus* species which are more reliable than the CGB test alone. It is also important to point out that the application of PCR is about six days faster than that of the CGB test. Speed is important due to the severity of illness and urgency in beginning the treatment as early as possible. The results suggest that the multiplex PCR is more reliable and faster than the CGB test, providing a trustworthy species identification which can be used as a complementary method to confirm the CGB results and avoiding possible diagnostic mistakes caused by false positive and negative which may occasionally be generated by the CGB test. Moreover, the multiplex PCR proved to be species-specific and is sufficiently sensitive to be applied in clinical laboratories.

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References

1 Idnurm A, Bahn YS, Nielsen K, *et al*. Deciphering the model pathogenic fungus *Cryptococcus neoformans*. *Nat Rev Microbiol* 2005; **3**: 753–764.

2 Kwon-chung KJ, Boekout T, Fell JW, Diaz M. Proposal to conserve the name *Cryptococcus gatti* against *C. hondurianus* and *C. bacillisporus*. *Taxon* 2002; **51**: 804–806.

3 Kidd SE, Bach PJ, Hingston AO, *et al*. *Cryptococcus gattii* dispersal mechanism, British Columbia, Canada. *Emerg Infect Dis* 2007; **13**: 51–57.

4 MacDougall L, Fyfe M. Emergence of *Cryptococcus gattii* in a novel environment provides clues to its incubation period. *J Clin Microbiol* 2006; **44**: 1851–1852.

5 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.

6 Stephen C. Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. *Can Vet J* 2002; **43**: 792–794.

7 Sorrel TC. *Cryptococcus neoformans* variety *gattii*. *Med Mycol* 2001; **39**: 155–168.

8 Trilles L, Fernandez-Torres B, Lazera MR, Wanke B, Guarro J. *In vitro* antifungal susceptibility of *Cryptococcus gattii*. *J Clin Microbiol* 2004; **42**: 4815–4817.

9 Kwon-Chung KJ, Polacheck I, Bennett J. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). *J Clin Microbiol* 1982; **15**: 535–537.

10 Abegg MA, Cella FL, Faganello J, *et al*. *Cryptococcus neoformans* and *Cryptococcus gattii* isolated from the excreta of Psittaciformes in a southern brazilian zoological garden. *Mycopathologia* 2006; **161**: 83–91.

11 Horta JA, Staats CC, Casali AK, *et al*. Epidemiological aspects of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *Med Mycol* 2002; **40**: 565–571.

12 Canelo C, Navarro A, Guevara M, *et al*. Determinación de la variedad de cepas de *Cryptococcus neoformans* aisladas de pacientes con SIDA. *Rev Med Exp* 1999; **15**: 44–47.

13 Nakamura Y, Kano R, Sato H, *et al*. Isolates of *Cryptococcus neoformans* serotype A and D developed on canavanine-glycine-bromothymol blue medium. *Mycoses* 1998; **41**: 35–40.

14 Khan ZU, Al-Alnezi AA, Chandy R, Xu J. Disseminated cryptococcosis in an AIDS patient caused by a canavanine-resistant strain of *Cryptococcus neoformans* var. *grubii*. *J Med Microbiol* 2003; **52**: 271–275.

15 Aoki FH, Amai T, Tanaka R, *et al*. New primer pairs specific for *Cryptococcus neoformans* serotype A or B prepared on the basis of random amplified polymorphic DNA fingerprinting pattern analyses. *J Clin Microbiol* 1999; **37**: 315–320.

16 Casali AK, Goulart L, Rosa e Silva LK, *et al*. Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *FEMS Yeast Res* 2003; **3**: 405–415.

17 Latouche GN, Huynh M, Sorrel TC, Meyer W. PCR-Restriction Fragment Length Polymorphism analysis of the phospholipase B (PLB1) gene for subtyping of *Cryptococcus neoformans* isolates. *Appl Environ Microbiol* 2003; **69**: 2080–2085.

18 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.

19 Diaz MR, Boekhout T, Kiesling T, Fell JW. Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast *Cryptococcus neoformans*. *FEMS Yeast Res* 2005; **5**: 1129–1140.

20 Kabasawa K, Itagaki H, Ikeda R, *et al*. Evaluation of a new method for identification of *Cryptococcus neoformans* which uses serologic tests aided by selected biological tests. *J Clin Microbiol* 1991; **29**: 2873–2876.

21 Zhang D, Yang Y, Castlebury LA, Cerniglia CE. A method for the large scale isolation of high transformation. *FEMS Microbiol Lett* 1996; **145**: 261–265.

22 Chaturvedi S, Dyavaiah M, Larsen RA, Chaturvedi V. *Cryptococcus gattii* in AIDS patients, Southern California. *Emerg Infect Dis* 2005; **11**: 1686–1692.

23 Meyer W, Castañeda A, Jackson S, *et al*. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis* 2003; **9**: 189–195.

ANEXO 2

FUENTEFRIA, A. M.; SUH, S-O.; LANDELL, M. F.; FAGANELLO, J.; SCHRANK, A.; VAINSTEIN, M. H.; BLACKWELL, M. & VALENTE, P.

***Trichosporon insectorum* sp. nov., a new anamorphic basidiomycetous killer yeast.**

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Trichosporon insectorum sp. nov., a new anamorphic basidiomycetous killer yeast

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ABSTRACT

Three killer yeasts, isolated from the gut of insects in Panama and artisanal cheese in Brazil, were shown to be related to the Ovoides clade of the genus *Trichosporon*. Sequencing of the D1/D2 region of the LSU rDNA and physiological characterization revealed a distinct taxonomic position in relation to known species of the genus. Conspecificity of the three killer isolates was reinforced by similar M13 fingerprinting and killer profiles. We propose a new species in this genus: *Trichosporon insectorum*. The type strain is CBS 10422^{T} (syn. NRRL Y-48120). This anamorphic species produces arthroconidia but not appressoria, and its killer character seems to be associated with dsRNA.

Introduction

Trichosporon species are basidiomycetous yeasts (*Hymenomycetes*, *Tremelloidae*, *Trichosporonales*) without a known sexual state (Middelhoven et al. 2004). They are characterized morphologically by production of septate mycelia and arthroconidia, although certain species possess additional morphological characters, such as formation of appressoria or macroconidia (Guého et al. 1992; Middelhoven et al. 2004). Some species are commonly recognized as opportunistic pathogens *viz.*: *T. asahii*, *T. asteroides*, *T. cutaneum*, *T. inkin*, *T. mucoides* and *T. ovoides* (Guého et al. 1998; Sugita et al. 1996). Other species are isolated from substrates not related to clinical samples (Middelhoven 2004; Middelhoven et al. 2004; Sugita et al. 2002a). Several new *Trichosporon* species have been recently described, e.g. *T. mycotoxinivorans* isolated from the hindgut of termites (Molnar et al. 2004).

The genus *Trichosporon* is monophyletic. Based on nuclear base sequencing of the SSU rDNA (Guého et al. 1992; Sugita & Nakase 1998) the genus could be divided into three groups of different species, although more groups are recognized in the *Trichosporonales* order (Okoli et al. 2006; Takashima et al. 2001). Sequence analyses of the D1/D2 region of the LSU rDNA (Guého et al. 1998; Fell et al. 2000) and ITS region (Scorzetti et al. 2002; Sugita et al. 1999) supported division of *Trichosporon* species into four clades. Middelhoven et al. (2004) proposed the names

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Gracile, Porosum, Cutaneum and Ovoides to designate these groups. In addition to rDNA base sequencing, traditional physiological characterization and serotyping, *Trichosporon* clades and species can be distinguished by their assimilation of several organic compounds not traditionally used in routine metabolic tests (Middelhoven 2002, 2004), and sequencing of the mitochondrial cytochrome b genes (Biswas *et al.* 2005) and IGS regions (Sugita *et al.* 2002b).

In the course of two independent studies, two yeast strains isolated from the gut of beetles in Panama and one from artisanal cheese in Brazil were found to belong to a new species of *Trichosporon*. Here we propose the new species, *T. insectorum*, based on phylogenetic analysis using sequences of the SSU rDNA and the D1/D2 region of LSU rDNA as well as morphological and physiological characteristics. Additional support for conspecificity of the three isolates was provided by similar M13 fingerprints and killer activity.

Materials and methods

Strains examined

The strains BG02-6-9-2 (syn. CBS10421; NRRL Y-48121) and BG02-7-20-011B-1-2 (syn. CBS 10422^T, NRRL Y-48120^T) were isolated during a study of yeasts associated with the digestive tract of insects at Barro Colorado Island, Panama (Suh *et al.* 2005). The strain KYQU89 (syn. CBS10423, NRRL Y-48122) was isolated from Brazilian artisanal cheese (Landell *et al.* 2006). Cultures were maintained at 4 °C in GYMP (2 % glucose, 0.5 % yeast extract, 2 % malt extract, 0.2 % monobasic sodium phosphate, 2 % agar) agar slants covered with sterile mineral oil or kept frozen in YM broth (1 % glucose, 0.3 % malt extract, 0.3 % yeast extract, 0.5 % peptone) with 15 % glycerol.

Morphological, biochemical and physiological characteristics

Conventional yeast identification based on phenotypic characters was performed according to Yarrow (1998), using the keys provided by Barnett *et al.* (2000) and the computer program YEASTCOMPARE (Ciriello & Lachance 2001).

Evaluation of the killer phenomenon

Assessment of killer activity was performed in modified YM agar [YM broth containing 15 % glycerol, 2 % agar and 0.003 % methylene blue (Sigma, St Louis), buffered to pH 5 with citrate buffer). As sensitive strains, 30 clinical and environmental isolates of *Cryptococcus neoformans* and *C. gatti* were tested against the three *Trichosporon* strains. Strain CBS10423 was further tested against 70 yeasts, including *C. neoformans*/*C. gattii* isolates and 27 other yeast strains belonging to several species (e.g., *Candida albicans*, *C. dubliniensis*, *C. parapsilosis*, *C. glabrata*, *C. catenulata*, *C. krusei*, *C. tropicalis* and *Yarrowia lipolytica*). Overnight grown target cells from sensitive yeasts were suspended in distilled water to a density of 4×10^5 cells ml^{-1}, and were spread on the assay Petri dishes. Yeast killer strains were point inoculated over the sensitive yeast inoculum, and plates were incubated at 25 °C for 72 h. The strain was considered to be a killer if there was an evident zone of inhibition of the sensitive yeast around its inoculum.

Characterization of the killer phenomenon

Total nucleic acid of strain CBS10423 was isolated as described by Ramos *et al.* (2001) from 3-d-old cultures, separated by electrophoresis on a 0.8 % agarose gel with ethidium bromide and visualized under UV light. Characterization of the high mobility nucleic acid bands was performed by treatment with the enzymes S1 nuclease and RNase, which cleave single-stranded nucleic acids and double-stranded RNA, respectively, according to Golubev *et al.* 2003, followed by 0.8 % agarose gel electrophoresis. Enzymatic treatments were carried out for 30 min at 37 °C, as follows: 1 unit S1 nuclease (Sigma) and 50 μg ml^{-1} RNase (Sigma). Aliquots of 0.1 ml of strain CBS10423 cells diluted in water (10^5 cell ml^{-1}) were spread on YEPD agar (2 % glucose, 1 % peptone, 0.5 % yeast extract, 2 % agar) plates and incubated at 42 °C (maximum growth temperature) for two weeks in order to induce thermal cure. Single colonies were selected at random and assayed for their killer activity. Colonies that lost the killer character had their total nucleic acid extracted and analysed as described above.

DNA sequencing and phylogenetic analysis

DNA extraction and amplification of the D1/D2 region of the LSU rDNA were performed for isolate CBS10423 as described in Ramos *et al.* (2001), using the primers NL_1-5′GCATATCAATAAGCGGAGGAAAAG3′ and NL_4-5′GGTCCGTGTTTCAAGACGG3′ (O'Donnell 1993). The purified PCR product (100 ng) was used for sequencing with the primers NL1 and NL4. DNA sequencing reactions were performed using a DYEnamic ET dye terminator cycle sequencing (MegaBACE) kit (Amersham Biosciences, Piscataway, New Jersey) and run on MegaBACE 1000 capillary sequencers. DNA sequencing for the other two strains, CBS10421 and CBS10422, was performed as described in Suh *et al.* (2005). Sequences were assembled using phred/phrap/consed (http://www.phrap.org) and analysed with the Nucleotide-nucleotide BLAST (blastn) program (Altschul *et al.* 1997), available at http://www.ncbi.nlm.nih.gov/BLAST/. DNA sequences were aligned using the CLUSTAL X computer program (Thompson *et al.* 1997) and optimized visually. Phylogenetic trees were constructed using NJ analysis using MEGA version 3.0 (Kumar *et al.* 2001). *Cryptococcus humicola* CBS 571 and *Filobasidiella neoformans* CBS132 were used as outgroup taxa. BS confidence values were based on 1000 replications.

M13 PCR fingerprinting

The oligonucleotide of the minisatellite-specific core sequence of the wild-type phage M13 (5′-GAG GGT GGC GGT TCT-3′) was used as primer for PCR reactions, according to Meyer *et al.* (1999). Thirty-five cycles of 20 s at 94 °C, 1 min at 50 °C and 20 s at 72 °C were performed, followed by a single extension cycle at 72 °C for 6 min. Amplified products were separated by electrophoresis in 1.8 % agarose gels for 5 h at 60 V, and were visualized under UV light after staining with ethidium bromide.

Taxonomy

Trichosporon insectorum A.M Fuentefria, S.O. Suh, M.F. Landell, J. Faganello, A. Schrank, M.H. Vainstein, M. Blackw & P. Valente, **sp. nov.** (Figs 1–3)

MycoBank no.: MB510822

Etym.: The name *T. insectorum* was chosen in honour of the habitat of the first two strains isolated (CBS 10421 and CBS 10422).

In liquido 'YM', post dies 5 ad 25 °C, cellulae ovoideae, ellipsoideae, elongatae, 5–10 × 3–4 μm, singulae aut binae. Sedimentum formatur. Post unum mensem ad 25 °C, pellicula completa et sedimentum formantur. In agaro 'YM', post unum mensem ad 25 °C, cultura cremea, butyracea, margo fimbriata. Arthroconidia, pseudomycelium et mycelium formantur neque appressoria. Glucosum non fermentatur. Glucosum, galactosum, L-sorbosum (exigue), D-glucosaminum (exigue), D-ribosum, D-xylosum, L-arabinosum, sucrosum, maltosum, trehalosum, cellobiosum, salicinum (lente), arbutinum, lactosum, melezitosum (exigue), xylitolum (exigue), inositolum, D-gluconatum, D-glucuronatum, butano-2, 3-diolum (lente), ethanolum, propane-1, 2-diolum (lente), D-glucaratum (lente), glycerolum, acidum succinicum et 2-keto-D-gluconatum assimilantur. D-arabinosum (vel exigue), L-rhamnosum, raffinosum (vel exigue), ribitolum (vel lente), D-mannitolum (vel lente), melibiosum, inulinum, amylum solubile, D-glucitolum, galactitolum, gluconolactonum, DL-acidum lacticum, citratum, methanolum, acidum quinicum et erythritolum non assimilantur. Aethylaminum (variabile), L-lysinum, cadaverinum et D-tryptophanum (exigue) assimilantur. kali nitratum, sodii nitritum, creatinum, creatininum, glucosaminum et imidazolum non assimilantur. 42 °C crescit neque 45 °C. Ureum finditur. Diazonium caeruleum B positivum. Crescere potest in medio 100 mg ml^{-1} cycloheximido addito. Materia amyloidea formatur.

Typus: Panama: *Barro Colorado Island*, isolata a ile *Eurysternys* sp. (*Coleoptera: Scarabaeidae*). BG 02-7-20-011B-1-2 (CBS 10422^{T} — holotypus; NRRL Y-48120 — isotypus).

The yeasts showed the typical morphological characteristics of the genus *Trichosporon* after 4 d at 25 °C in YM broth: cells are ovoidal, ellipsoidal, elongated, 5–10 × 3–4 μm, single or in pairs (Fig 1A) with sediment formed. After one month at 25 °C a complete pellicle and sediment are present. On YM agar after one month at 25 °C the streak culture is cream-coloured, butyrous with a fimbriate margin. Dalmau plate culture on corn meal agar for 4 d at 25 °C shows development of pseudohyphae and/or septate hyphae (Fig 1B) and arthroconidia (Fig 1C). Appressoria are absent. Sexual reproduction was not observed. Physiological characteristics are summarized in Table 1.

Other specimens examined: Panama: *Barro Colorado Island*, isolated from the gut of *Paxillus leachi* (*Coleoptera: Passalidae*), BG 02-6-9-2 (CBS 10421; NRRL Y-48121) — Brazil: *Rio Grande do Sul*, isolated from artisanal cheese (CBS 10423; NRRL Y-48122).

Results and discussion

The results of morphological and physiological tests indicated that the three isolates, CBS 10421, CBS 10422 and CBS 10423, belong to the genus *Trichosporon*, but they were clearly distinguished from all other species of the genus (Table 1, Fig 1). Phylogenetic trees based on the D1/D2 sequences (Fig 2) and SSU rDNA sequences (data not shown) placed this new species in the Ovoides clade of the genus *Trichosporon*. The DNA sequences of the D1/D2 region were identical among the three isolates, and they differed from the closely related species *T. faecale*, *T. asteroides*, *T. asahii* and *T. japonicum* by more

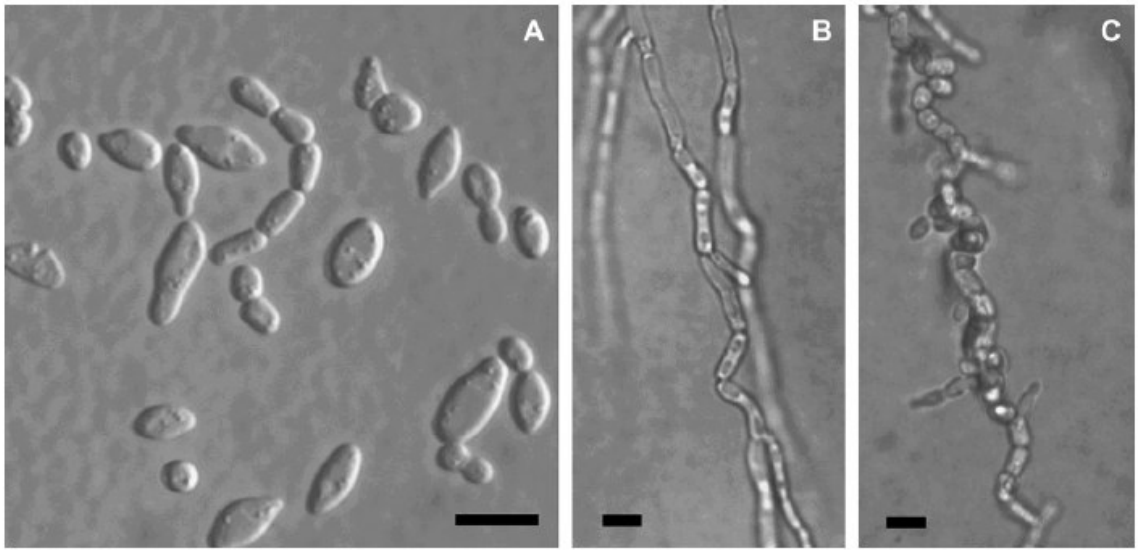

Fig 1 – Morphology of *Trichosporon insectorum* CBS 10422^{T}. (A) Various shapes of vegetative cells and arthroconidia grown in YM broth for 4 d at 25 °C. (B) Hyphae on corn meal agar after for 4 d at 25 °C. (C) Hyphae disarticulating into arthroconidia on corn meal agar for 4 d at 25 °C. Bars = 10 μm.

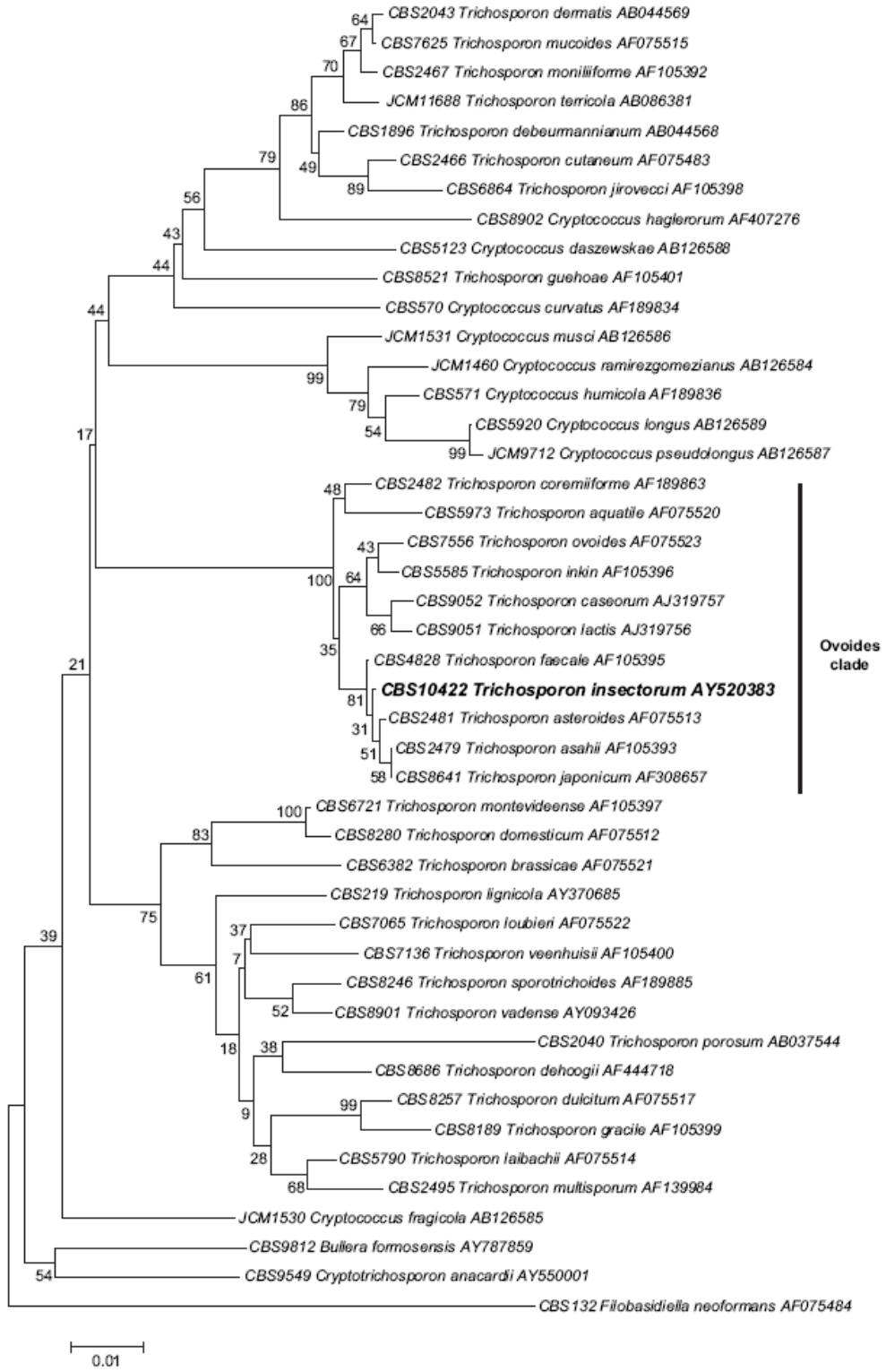

Fig 2 – Phylogenetic tree of **Trichosporon insectorum** sp. nov. and related taxa. The tree was constructed using the NJ method from D1/D2 26S rDNA sequences. The numerals represent the confidence level from 1000 replicates BS sampling.

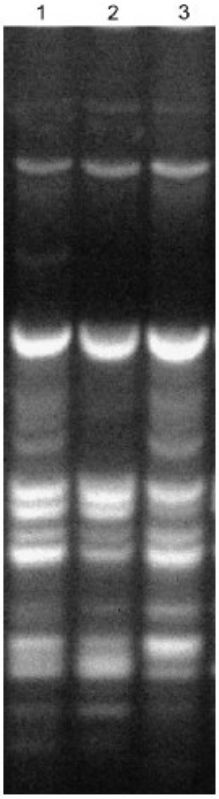

Fig 3 – PCR-fingerprinting patterns obtained with primers M13 from *Trichosporon insectorum* isolates. Lanes: 1, CBS 10423; 2, CBS 10422; 3, CBS 10421.

than two substitutions, which is generally considered enough genetic difference to separate species (Fell *et al.* 2000). In addition, differences in the biochemical results support the proposal of the new species, *T. insectorum*. Phenotypically, the new species is clearly distinguished from all species of the Ovoides clade by the inability to assimilate lactate and/or by delayed growth at 42 °C (Table 1).

The killer profile of strain CBS 10423 was assayed against a total of 100 isolates of *Cryptococcus neoformans*/*C. gattii*, showing 96 % inhibition of them. The four isolates that were not inhibited by strain CBS 10423 belonged to the species *C. gattii*. Strains belonging to clinically relevant *Candida* species were weakly inhibited, while there was no inhibition of *Yarrowia lipolytica* isolates. Assessment of the killer profile of strains CBS 10421 and CBS 10422 against 30 isolates of *C. neoformans*/*C. gattii* showed the same results as those obtained with strain CBS 10423 (data not shown). The killer phenomenon has already been reported for other *Trichosporon* species (Golubev 2006), and seems to be a common character in the genus. Several authors have reported the inhibition of isolates of *C. neoformans* by killer yeasts (Boekhout & Scorzetti 1997; Buzzini & Martini 2000; Cenci *et al.* 2004; Criseo *et al.* 1999; Fuentefria *et al.* 2006; Morace *et al.* 1984), but few mentioned the inhibition of such a wide variety of isolates as used in the present study.

Table 1 – Biochemical and physiological characterization of *Trichosporon insectorum* sp. nov.

Fermentation of carbon compounds			
D-Glucose			–
Assimilation of carbon compounds			
D-Glucose	+	Ribitol	–, D
D-Galactose	+	Xylitol	W
L-Sorbose	W	D-Glucitol	–
D-Glucosamine	W	D-Mannitol	–, D
D-Ribose	+	Galactitol	–
D-Xylose	+	myo-Inositol	+
L-Arabinose	+	D-Glucono-1,5-lactone	–
D-Arabinose	–, W	2-Keto-D-gluconate	+
L-Rhamnose	–	D-Gluconate	+
Sucrose	+	D-Glucuronate	+
Maltose	+	DL-Lactate	–
Trehalose	+	Succinate	+
Cellobiose	+	Citrate	–
Salicin	D	Methanol	–
Arbutin	+	Ethanol	+
Melibiose	–	Propane 1,2 diol	D
Lactose	+	Butane 2,3 diol	D
Raffinose	–, W	Quinic acid	–
Melezitose	W	D-Glucarate	D
Inulin	–	Glycerol	+
Soluble starch	–	Erythritol	–
Assimilation of nitrogen compounds			
Nitrate (potassium)			–
Nitrite (sodium)			–
Ethylamine			V
L-Lysine			+
Cadaverine			+
Creatine			–
Creatinine			–
D-Glucosamine			–
Imidazole			–
D-Tryptophan			W
Growth at			
25 °C			+
30 °C			+
37 °C			+
40 °C			+
42 °C			D
45 °C			–
Vitamin requirement			
w/o Pantothenate			+
w/o Biotin			–
w/o Thiamin			+
w/o Biotin & Thiamin			–
w/o Pyridoxine			+
w/o Pyridoxine & Thiamine			+
w/o Niacin			+
w/o PABA			+
Other additional tests			
0.01 % Cycloheximide			+
1 % Acetic acid			–
50 % D-Glucose			V
60 % D-Glucose			–
10 % Sodium chloride			V
16 % Sodium chloride			–, W
Starch formation			+
Urea hydrolysis			+
Diazonium blue B reaction			+

(+) Growth within 21 d; (–) no growth after 21 d; (v) variable response; (D) growth after 21 d or more.

Attempts to cure the killer character of strain CBS 10423 by cultivation at the maximum growth temperature resulted in loss of the killer character in approximately 10 % of the colonies. The cured colonies did not present the smaller nucleic acid bands typical of the original strain when total nucleic acid content was analysed using 0.8 % agarose gel electrophoresis, and only presented the low-mobility band corresponding to chromosomal DNA molecules. This loss of the smaller bands was also achieved when the nucleic acid of the original CBS 10423 strain was treated with RNase, demonstrating that the bands corresponded to dsRNA. Treatment with S1 nuclease was innocuous. Therefore, the killer character in *Trichosporon insectorum* seems to be associated with dsRNA molecules.

M13 PCR fingerprinting was performed with the objective of confirming the genetic relatedness among the isolates obtained from beetle guts and cheese, because strains that belong to the same species have similar M13 fingerprint profiles (Sampaio *et al.* 2001). The fingerprint patterns of isolates CBS 10422, CBS 10421, and CBS 10423 were nearly identical (Fig 3). The results from the phylogenetic analysis, DNA sequence comparisons, M13 fingerprint profile, and killer pattern support the hypothesis that the three isolates are conspecific.

It is tempting to hypothesize that the presence of strain CBS10423 in cheese is due to contamination of arthropod origin, especially because it was isolated from artisanal cheese marketed at stands with poor hygienic conditions. In fact certain mites are well known for their infestation of cheeses, even to the extent that they cause work place allergies (Gorham 1979). However, a connection with beetles in the present case awaits additional information on the ecology of *T. insectorum*. Other members of the Ovoides clade, e.g. *T. caseorum* and *T. lactis*, also have been isolated from cheese (Lopandic *et al.* 2004).

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REFERENCES

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25: 3389–3402.

Barnett JA, Payne RW, Yarrow D, 2000. *Yeasts: Characteristics and Identification*, third edn. Cambridge University Press, Cambridge.

Biswas SK, Wang L, Yokoyama K, Nishimura K, 2005. Molecular phylogenetics of the genus *Trichosporon* inferred from mitochondrial cytochrome b gene sequences. *Journal of Clinical Microbiology* 43: 5171–5178.

Boekhout T, Scorzetti G, 1997. Differential killer toxin sensitivity patterns of varieties of *Cryptococcus neoformans*. *Journal of Medical and Veterinary Mycology* 35: 147–149.

Buzzini P, Martini A, 2000. Biodiversity of killer activity in yeasts isolated from Brazilian rain Forest. *Canadian Journal of Microbiology* 46: 607–611.

Cenci E, Bistoni F, Mencacci A, Perito S, Magliani W, Conti S, Polonelli L, Vecchiarelli A, 2004. A synthetic peptide as a novel anticryptococcal agent. *Cellular Microbiology* 6: 953–961.

Ciriello CJ, Lachance MA, 2001. *YeastCompare*. University of Western Ontario, London.

Criseo G, Gallo M, Pernice A, 1999. Killer activity at different pHs against *Cryptococcus neoformans var. neoformans* serotype A by environmental yeast isolates. *Mycoses* 42: 601–608.

Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A, 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *International Journal of Systematic and Evolutionary Microbiology* 3: 1351–1371.

Fuentefria AM, Franskoviaki IM, Mercado LW, Ramos JP, Vainstein MH, Valente P, 2006. Inhibition of clinical and environmental *Cryptococcus neoformans* isolates by two Brazilian killer yeasts. *Journal of Basic Microbiology* 46: 87–93.

Golubev WI, 2006. Antagonistic interactions among yeasts. In: Rosa CA, Peter G (eds), *Biodiversity and Ecophysiology of Yeasts*. Springer-Verlag, Berlin, pp. 197–219.

Golubev WI, Pfeiffer I, Churkina LG, Golubeva EW, 2003. Double-stranded RNA viruses in a mycocinogenic strain of *Cystofilobasidium infirmominiatum*. *FEMS Yeast Research* 3: 63–68.

Gorham JR, 1979. Significance for human health of insects in food. *Annual Review of Entomology* 24: 209–224.

Guého E, Smith MT, de Hoog GS, Billon-Grand G, Christen R, Batenburg-van der Vegte WH, 1992. Contributions to a revision of the genus *Trichosporon*. *Antonie van Leeuwenhoek* 61: 289–316.

Guého E, Smith MT, de Hoog GS, 1998. *Trichosporon* behrend. In: Kurtzman CP, Fell JW (eds), *The Yeasts, A Taxonomic Study*, 4th edn. Elsevier, Amsterdam, pp. 854–872.

Kumar S, Tamura K, Jakobsen IB, Nei M, 2001. *MEGA2: Molecular Evolutionary Genetics Analysis Software*. Arizona State University, Arizona.

Landell MF, Hartfelder C, Valente P, 2006. Identification and enzymatic profile of yeasts isolated from artisanal cheese in Southern Brazil. *Acta Scientiae Veterinariae* 34: 49–55.

Lopandic K, Sugita T, Middelhoven WJ, Herzberg M, Fell JW, Zelger S, Prillinger H, 2004. *Trichosporon caseorum* sp. nov. and *Trichosporon lactis* sp. nov., two basidiomycetous yeasts isolated from cheeses. In: Agerer R, Piepenbring M, Blanz P (eds), *Frontiers in Basidiomycota Mycology*. IHW-Verlag, Eching, pp. 99–116.

Meyer W, Marszewska K, Amirmostofan M, Igreja RP, Hardtke C, Methling K, Viviani MA, Chindamporn A, Sukroon-Greung S, John MA, Ellis DH, Sorrell TC, 1999. 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* 20: 1790–1799.

Middelhoven WJ, 2002. Identification of clinically relevant *Trichosporon* species. *Mycoses* 46: 7–11.

Middelhoven WJ, 2004. *Trichosporon wieringae* sp. nov., an anamorphic basidiomycetous yeast from soil, and assimilation of some phenolic compounds, polysaccharides and other non-conventional carbon sources by saprophytic *Trichosporon* species. *Antonie van Leeuwenhoek* 86: 329–337.

Middelhoven WJ, Scorzetti G, Fell JW, 2004. Systematics of the anamorphic basidiomycetous yeast genus *Trichosporon*

Behrend with the description of five novel species: *Trichosporon vadense*, *T. smithiae*, *T. dehoogii*, *T. scarabaeorum* and *T. gamsii*. *International Journal of Systematic and Evolutionary Microbiology* **54**: 975–986.

Molnar O, Schatzmayr G, Fuchs E, Prillinger H, 2004. *Trichosporon mycotoxinivorans* sp. nov., a new yeast species useful in biological detoxification of various mycotoxins. *Systematic and Applied Microbiology* **27**: 661–671.

Morace G, Archibusacci C, Sestito M, Polonelli L, 1984. Strain differentation of pathogenic yeasts by the killer system. *Mycopathologia* **84**: 81–85.

O'Donnell K, 1993. *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds), *The Fungal Holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics*. CAB International, Wallingford, pp. 225–233.

Okoli I, Oyeda CA, Kwon-Chung KJ, Theelen B, Robert V, Groenewald JZ, McFadden DC, Casadevall A, Boekhout T, 2007. *Cryptotrichosporon anacardii* gen. nov., sp. nov., a new trichosporonoid capsulate basidiomycetous yeast from Nigeria that is able to form melanin on niger seed agar. *FEMS Yeast Research* **7**: 339–350.

Ramos JP, Valente P, de Souza RA, Rosa CA, Leoncini O, 2001. Heteroduplex mobility assay of the D1/D2 region of the 26s rDNA for differentiation of *Saccharomyces* species. *Letters in Applied Microbiology* **33**: 206–210.

Sampaio JP, Gadanho M, Santos S, Duarte FL, Pais C, Fonseca A, Fell JW, 2001. Polyphasic taxonomy of the basidiomycetous yeast genus *Rhodosporidium*: *Rhodosporidium kratochvilovae* and related anamorphic species. *International Journal of Systematic and Evolutionary Microbiology* **51**: 687–697.

Scorzetti G, Fell JW, Fonseca A, Statzell-Tallman A, 2002. Systematics of basidiomycetous yeasts: a comparison of large sub-unit D1D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Research* **2**: 495–517.

Sugita T, Nakase T, 1998. Molecular phylogenetic study of the basidiomycetous anamorphic yeast genus *Trichosporon* and related taxa based on small subunit ribosomal DNA sequences. *Mycoscience* **39**: 7–13.

Sugita T, Nishikawa A, Shinoda T, Kusunoki T, 1996. Taxonomic studies on clinical isolates from superficial trichosporonosis patients by DNA relatedness. *Japanese Journal of Medical Mycology* **37**: 107–110.

Sugita T, Nishikawa A, Ikeda R, Shinoda T, 1999. Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. *Journal of Clinical Microbiology* **37**: 1985–1993.

Sugita T, Takashima M, Nakase T, Ichikawa T, Shinoda T, Nishikawa A, 2002a. A basidiomycetous anamorphic yeast, *Trichosporon terricola* sp. nov., isolated from soil. *Journal of General and Applied Microbiology* **48**: 293–297.

Sugita T, Nakajima M, Ikeda R, Matsushima T, Shinoda T, 2002b. Sequence analysis of the ribosomal DNA intergenic spacer 1 regions of *Trichosporon* species. *Journal of Clinical Microbiology* **40**: 1826–1830.

Suh S-O, McHugh JV, Pollock DD, Blackwell M, 2005. The beetle gut: a hyperdiverse source of novel yeasts. *Mycological Research* **109**: 261–265.

Takashima M, Sugita T, Shinoda T, 2001. Reclassification of the *Cryptococcus humicola* complex. *International Journal of Systematic and Evolutionary Microbiology* **51**: 2199–2210.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. *Nucleic Acids Research* **25**: 4876–4882.

Yarrow D, 1998. Methods for the isolation, maintenance, and identification of yeasts. In: Kurtzman CP, Fell JW (eds), *The Yeasts, A Taxonomic Study*, 4th edn. Elsevier, Amsterdam, pp. 77–100.

ANEXO 3

FUENTEFRIA, A. M.; FAGANELLO, J.; PAZZINI, F.; SCHRANK, A.; VALENTE, P. & VAINSTEIN, M. Typing and patterns of cellular morphological alterations in *Cryptococcus neoformans* and *Cryptococcus gattii* isolates exposed to a panel of killer yeasts.

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Typing and patterns of cellular morphological alterations in *Cryptococcus neoformans* and *Cryptococcus gattii* isolates exposed to a panel of killer yeasts

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Cryptococcus neoformans and *Cryptococcus gattii* are encapsulated basidiomycetous yeasts that cause meningoencephalitis. The action of killer yeasts on the growth of one hundred genotypically characterized *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii*, and *C. gattii* clinical and environmental isolates was evaluated. Killer studies were performed on yeast malt-methylene blue (YM-MB) agar Petri dishes, and a dendrogram was obtained based on a quantitative data matrix using the diameter of the inhibition halo. The cellular morphological characteristics of dead cells within the halo were observed by means of optical and scanning electron microscopy. There was no formation of pores on the cell surface of the sensitive cells in contact with the toxins, at least for *C. neoformans*. The sensitivity patterns of clinical and environmental isolates to the killer toxins demonstrated that there is correlation between killer sensitivity of *Cryptococcus* species or varieties and some of the killer strains. In this case, the isolates were discriminated using the killer sensitivity patterns, and this could be used as a complementary tool to PCR-fingerprinting in epidemiological studies.

Keywords Killer yeasts, typing, *Cryptococcus neoformans*, *Cryptococcus gattii*, scanning electron microscopy

Introduction

The *Cryptococcus neoformans* species complex comprises encapsulated basidiomycetous yeasts that are a major cause of fungal meningoencephalitis, mainly in immunocompromised individuals, e.g., AIDS patients [1]. *C. neoformans* was formerly composed of two varieties, i.e., *C. neoformans* var. *neoformans* (serotypes A, D, and the AD hybrid) and *C. neoformans* var. *gattii* (serotypes B and C) [2]. The latter was recently raised to species status, *Cryptococcus gattii* [3]. According to molecular studies based on RFLP analysis and sequencing data, *C. neoformans* serotype A was recognized as a new variety, *C. neoformans* var. *grubii* [4], whereas serotype D was restricted to *C. neoformans* var. *neoformans* [1].

Significant efforts have been invested in molecular typing techniques to attain a level of intraspecific discrimination between *C. neoformans* and *C. gattii* that would be significantly superior to serotyping. Polymerase chain reaction (PCR) fingerprinting and restriction fragment length polymorphism (RFLP) [5], amplified length polymorphism AFLP [6] and partial sequence analyses of the intergenic spacer region (IGS) [7,8] have shown considerable genetic divergence among species and varieties.

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In the last years, biotyping procedures based on phenotypic characteristics that could be used as epidemiological markers for differentiating these pathogenic species and varieties have also been developed [9–11]. The killer sensitivity profile was applied by Boekhout and Scorzetti [9], who were able to distinguish four patterns of sensitivity among 129 isolates of *C. neoformans* var. *neoformans* and six among 32 isolates of *C. gattii* using six basidiomycetous killer yeasts.

In the present study we have verified the action of 20 killer yeasts on the growth of 100 genotypically characterized clinical ($n=72$) and environmental ($n=28$) isolates of *C. neoformans* var. *neoformans* ($n=12$), *C. neoformans* var. *grubii* ($n=75$), and *C. gattii* ($n=13$). In addition, we have evaluated the application of the killer sensitivity profile as a typing method and have analyzed the effect of the killer toxins on the cellular morphology of the sensitive cells by means of optical and scanning electron microscopy.

Materials and methods

Strains

The 100 isolates of *C. neoformans* and *C. gattii* strains studied are listed in Table 1. All *Cryptococcus* strains were previously characterized into molecular types by M13 and $(GACA)_4$ PCR-fingerprinting [12,13]. The killer panel was composed by 20 strains selected from previous screening of 44 non-*Saccharomyces* killer strains obtained from phylloplane of *Hibiscus rosa-sinensis* (KYHB), phylloplane of Bromeliads (KYBI), raw milk (KYLV), raw goat milk (KYLC), cheese (KYQU) and fruits (KYF), in Brazil. For molecular identification of the killer yeasts, the D1/D2 domains of the 26S rDNA gene were sequenced using the primers NL_1-5′GCATATCAATAAGCGGAGGAAAAG3′ and NL_4-5′GGTCCGTGTTTCAAGACGG3′ [14]. DNA sequencing reactions were performed using the DYEnamic ET dye terminator cycle sequencing (MegaBACE) kit and run on MegaBACE 1000 capillary sequencers (Amersham Biosciences). Sequences were assembled using phred/phrap/consed (http://www.phrap.org) and analyzed with Nucleotide-nucleotide BLAST (blastn) program [15], available in the site http://www.ncbi.nlm.nih.gov/BLAST/. The killer strains *Kodamaea ohmeri* KYHB55 and KYHB88 were previously identified by sequencing of D1/D2 domains of the 26S rDNA gene [16]. Cultures were maintained at 4°C on glucose-yeast extract-malt extract-phosphate (GYMP; glucose 2%, yeast extract 0.5%, malt extract 2%, monobasic sodium phosphate 0.2%, agar 2%) agar slants covered with sterile mineral oil, and deposited at the yeast culture collection of the Microbiology Department of the Federal University of Rio Grande do Sul.

Killer assay

Overnight grown target cells from *C. neoformans* and *C. gattii* isolates were suspended in distilled water to a density of approximately 10^6 cells/mL and spread on the assay Petri dishes containing yeast-malt methylene blueagar (YM-MB; yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1%, agar 2% and methylene blue Sigma 0.003%, pH 5.5). Inoculum suspensions (10^8 cells/ml) of 24 h grown cells of potential killer yeasts were prepared, imprinted onto the sensitive yeast inoculum by the Steer's replicator, and incubated at 25°C for 3–5 days. The killer activity was considered positive if there was an evident zone of inhibition around the inoculum of the killer strain, surrounded by a crown of dark-blue stained cells. A narrow but clear inhibition zone was interpreted as a weak killer action.

Data matrix typing method

Using a metric ruler, the diameter of the zone of inhibition was measured in millimeters for each sensitive strain. As a result, a quantitative data matrix (QDM) was obtained [17]. All numerical data represent the average value of three separate determinations ($P<0.01$). Statistic evaluation of the data was obtained by ANOVA. A cluster analysis was performed for estimating the killer typing efficacy using a normalized Euclidean distance matrix based on the QDM data, through the statistical free software R Package, version 4.0 and Phylip, version 3.57. Neighbor joining clustering method was used to obtain the final dendrogram, with a 10,000 times resampling with replacement using a bootstrap procedure. A consensus tree was obtained using the CONSENSUS application from PHYLIP software.

Observation of morphological alterations in Cryptococcus cells exposed to the killer toxin

For the observation of morphological alterations *C. neoformans* var. *grubii* (isolates C95, AZ29), *C. neoformans* var. *neoformans* (C76, E14) and *C. gattii* (C40, C20) were used as sensitive strains, and *Trichosporon faecale* (KYQU100, KYQU88 and KYQU45), *Trichosporon japonicum* (KYQU139) and *Trichosporon coremiiforme* (KYQU83, KYLC83) as killer yeasts. The test was performed at 30°C for 48 h in separate experiments. The morphology of dead cells within the

Table 1 Quantitative Data Matrix (QDM) concerning the differential growth inhibition haloes of the *Cryptococcus neoformans* and *Cryptococcus gattii* isolates studied

Species/Varieties Strain number	Characteristics	Serotype/Molecular Type	Killer yeasts																		
			KYQU31	KYQU45	KYQU112	KYHB88	KYHB55	KYQU72	KYQU88	KYLY22	KYHB249	KYLY91	KYQU99	KYLY73	KYE802	KYLC60	KYQU139	KYLY102	KYQU127	KYQU83	KYQU100	KYLC83
C. gattii																					
(1) AZ13	Parrot droppings	B/VGI	C	C	E	E	B	D	D	B	A	D	C	A	A	A	E	C	A	D	C	D
(2) AZ14	Parrot droppings	B/VGI	C	C	E	E	B	D	D	B	A	C	C	A	A	A	E	C	A	D	C	C
(3) C2	Clinical isolate, HIV+	B/VGIII	C	C	E	B	C	E	E	B	A	D	C	C	C	D	E	C	C	E	D	D
(4) C20	Clinical isolate, HIV+	B/VGIII	B	C	D	B	B	D	D	A	A	D	A	B	D	D	D	B	C	A	D	B
(5) C40	Clinical isolate, Male	B/VGIII	B	C	E	B	C	D	E	B	A	C	C	B	D	D	E	C	C	E	E	D
(6) C41	Clinical isolate, Male	B/VGIII	B	C	E	D	C	B	D	C	A	B	B	D	A	A	E	C	A	A	E	D
(7) C42	Clinical isolate, Male, tumor	B/VGIII	A	D	D	C	A	E	E	A	A	B	B	C	D	B	C	B	A	E	A	B
(8) C43	Clinical isolate, Female	B/VGIII	C	B	E	B	C	E	E	B	A	D	D	C	C	D	E	D	C	E	D	D
(9) C44	Clinical isolate, Male, HIV+	B/VGIII	A	A	B	B	B	B	B	A	A	B	A	B	A	A	B	A	A	D	C	B
(10) C45	Clinical isolate, Male	B/VGIII	B	D	B	A	D	E	B	C	A	A	A	A	A	A	B	A	A	A	A	E
(11) C46	Clinical isolate, Female	B/VGIII	B	E	A	A	B	A	A	B	A	A	A	A	A	B	B	A	A	A	B	D
(12) C47	Clinical isolate, Male	B/VGIII	D	D	E	C	D	E	E	C	B	D	C	C	C	C	E	C	D	E	D	D
(13) C48	Clinical isolate, Male	B/VGIII	D	E	E	E	B	E	E	D	E	A	C	A	D	A	E	A	A	B	B	E
C. n. var. *grubii*																						
(1) E1	Pigeon droppings	A/VNI	D	A	D	F	A	E	E	A	A	B	A	A	D	B	G	B	A	E	E	B
(2) E2	Parakeet droppings	A/VNI	B	A	D	D	A	D	D	A	A	E	B	A	A	A	F	A	A	F	D	B
(3) E3	Pigeon droppings	A/VNI	A	A	E	F	B	D	E	A	A	E	A	B	D	D	E	A	A	E	D	D
(4) E4	Pigeon droppings	A/VNI	A	A	B	B	A	D	D	A	A	D	A	B	D	A	E	B	D	E	D	D
(5) E9	Pigeon droppings	A/VNI	D	D	D	G	B	E	G	D	B	F	B	E	D	D	E	D	B	E	D	E
(6) AZ1	Parakeet droppings	A/VNI	A	A	D	E	B	F	G	B	A	A	A	A	B	A	E	A	A	F	D	B
(7) AZ5	Parakeet droppings	A/VNI	B	A	F	E	B	D	D	D	A	F	B	B	B	B	E	B	B	E	D	B
(8) AZ6	Parrot droppings	A/VNI	A	B	D	D	B	G	F	A	A	D	B	B	E	B	F	C	D	G	D	C
(9) AZ9	Parakeet droppings	A/VNI	B	D	F	F	C	F	G	A	D	F	B	A	D	B	H	D	D	G	G	D
(10) AZ10	Parakeet droppings	A/VNI	B	B	D	E	B	F	F	A	A	D	B	D	E	B	D	D	D	E	E	D
(11) AZ15	Parakeet droppings	A/VNI	D	D	G	F	D	D	D	B	A	E	A	B	D	B	D	B	B	F	D	D
(12) AZ16	Parakeet droppings	A/VNI	D	B	F	F	B	D	E	D	B	E	B	D	D	B	E	B	B	G	D	E
(13) AZ18	Parakeet droppings	A/VNI	B	D	E	E	B	H	G	A	D	D	A	B	E	D	G	C	D	G	D	D
(14) AZ19	Parakeet droppings	A/VNI	B	D	F	E	A	G	F	A	A	D	A	B	F	D	H	D	C	F	E	D
(15) AZ24	Parakeet droppings	A/VNI	B	B	D	E	B	H	H	A	A	D	A	B	F	E	G	B	D	G	D	D
(16) AZ30	Parrot droppings	A/VNI	A	D	D	D	A	F	H	A	A	A	A	B	E	E	G	B	D	H	F	D
(17) C1	Clinical isolate, HIV+	A/VNI	B	B	B	B	B	D	D	A	A	B	B	A	D	B	E	B	D	B	C	D
(18) C3	Clinical isolate, HIV+	A/VNI	B	D	D	E	B	D	E	B	B	D	D	B	B	D	E	D	D	F	D	E
(19) C4	Clinical isolate,CSF, HIV+	A/VNI	B	D	D	D	B	H	G	B	A	D	B	D	E	D	F	A	D	H	E	B
(20) C5	Clinical isolate,CSF, HIV+	A/VNI	A	D	D	D	B	F	G	B	A	E	B	D	E	D	F	A	D	H	E	D
(21) C6	Clinical isolate,CSF, HIV+	A/VNI	A	D	D	D	A	G	G	B	A	C	A	D	D	B	F	B	D	H	E	D
(22) C7	Clinical isolate,CSF, HIV+	A/VNI	C	D	D	D	B	H	G	A	A	D	B	D	E	C	E	B	D	H	A	B
(23) C8	Clinical isolate,HIV+	A/VNI	D	A	D	B	B	A	B	D	A	B	A	B	A	A	D	A	D	E	A	B
(34) C9	Clinical isolate,CSF, HIV+	A/VNI	D	C	E	D	D	E	F	A	B	E	B	B	D	D	H	C	D	G	E	E
(25) C10	Clinical isolate,CSF, HIV+	A/VNI	C	D	D	B	A	G	F	B	B	E	A	D	E	D	F	A	D	H	D	C
(26) C11	Clinical isolate,CSF, HIV+	A/VNI	B	B	B	D	B	G	F	D	A	E	A	B	E	D	F	B	C	H	D	D
(27) C12	Clinical isolate,CSF, HIV+	A/VNI	B	D	B	B	B	F	G	A	B	D	B	B	E	D	E	B	B	H	F	B
(28) C13	Clinical isolate,CSF, HIV+	A/VNI	A	B	B	B	B	F	G	B	B	E	B	B	F	D	F	D	B	H	F	B
(29) C14	Clinical isolate,CSF, HIV+	A/VNI	A	D	D	B	B	H	G	D	B	E	D	B	E	D	F	A	D	G	E	D

Table 1 (*Continued*)

Species/Varieties Strain number	Characteristics	Serotype/Molecular Type	KVQU31	KVQU45	KVQU112	KVHB88	KVHB55	KVQU72	KVQU88	KVLV22	KVBI249	KVLV91	KVQU99	KVLV73	KVE002	KVLC60	KVQU139	KVLV102	KVQU127	KVQU83	KVQU100	KVLC83
(30) C15	Clinical isolate,CSF, HIV+	A/VNI	B	D	D	B	B	F	H	B	A	D	C	D	E	B	F	B	D	G	D	D
(31) C16	Clinical isolate,CSF, HIV+	A/VNI	A	B	D	D	A	H	G	D	A	D	B	D	E	B	E	B	D	F	D	D
(32) C17	Clinical isolate,CSF, HIV+	A/VNI	D	B	D	D	A	H	G	D	A	E	B	D	F	D	E	A	D	G	E	D
(33) C18	Clinical isolate,HIV+	A/VNI	B	D	D	A	B	D	B	A	A	D	B	B	A	A	B	A	B	E	B	B
(34) C19	Clinical isolate,HIV+	A/VNI	B	D	B	A	A	B	B	A	A	A	A	A	A	A	B	A	B	B	A	A
(35) C27	Clinical isolate,Female, HIV+	A/VNI	D	D	E	E	B	E	G	B	E	F	D	D	E	D	F	E	D	F	E	F
(36) C29	Clinical isolate,Female, HIV+	A/VNI	B	B	D	B	A	D	D	A	A	D	A	A	B	A	D	B	D	D	D	B
(37) C31	Clinical isolate,Female, HIV+	A/VNI	A	A	E	F	A	E	H	D	A	E	A	A	E	A	G	A	A	G	F	B
(38) C32	Clinical isolate,Female, HIV+	A/VNI	B	A	D	H	B	D	D	B	B	D	A	B	D	D	F	E	D	F	D	G
(39) C33	Clinical isolate,Male, HIV +	A/VNI	B	B	F	D	B	B	B	A	A	B	D	B	B	B	F	D	B	B	F	D
(40) C34	Clinical isolate,Male	A/VNI	D	D	F	H	D	E	G	B	B	E	B	D	D	D	E	D	E	G	D	F
(41) C49	Clinical isolate,Male, HIV +	A/VNI	B	D	E	D	B	F	H	D	B	B	B	B	F	D	H	B	D	G	E	E
(42) C50	Clinical isolate,Female, HIV+	A/VNI	A	A	E	B	A	D	D	B	A	E	A	A	A	A	F	A	A	D	C	B
(43) C51	Clinical isolate,Male, HIV +	A/VNI	A	D	D	B	A	H	G	A	D	A	A	D	G	D	E	D	D	H	D	B
(44) C52	Clinical isolate,Male, HIV +	A/VNI	B	B	F	E	B	H	F	B	B	B	D	D	F	D	H	G	D	E	F	D
(45) C53	Clinical isolate,Male, HIV +	A/VNI	D	D	G	F	C	H	D	A	D	D	B	D	G	D	F	D	D	D	F	D
(46) C54	Clinical isolate,Male, HIV +	A/VNI	A	D	E	D	A	G	H	A	A	D	A	D	F	E	H	F	E	F	F	D
(47) C55	Clinical isolate,Female, HIV+	A/VNI	B	D	D	E	B	F	H	D	D	D	A	B	F	E	E	F	D	H	D	D
(48) C56	Clinical isolate,Male, HIV +	A/VNI	B	D	F	D	B	F	G	B	A	D	D	A	G	D	G	F	D	G	E	B
(49) C57	Clinical isolate,Male, HIV +	A/VNI	A	B	B	C	B	G	G	A	A	E	B	A	F	D	E	G	D	G	F	B
(50) C58	Clinical isolate,Male, HIV +	A/VNI	A	A	E	D	B	G	H	C	A	D	D	B	F	E	F	D	D	F	F	D
(51) C59	Clinical isolate,Male, HIV +	A/VNI	A	B	B	B	A	H	G	D	D	B	A	D	F	E	H	E	B	G	D	E
(52) C60	Clinical isolate,Male, HIV +	A/VNI	B	C	F	F	B	G	F	B	B	A	D	D	F	D	H	F	B	F	D	E
(53) C61	Clinical isolate,Male, HIV +	A/VNI	C	D	G	E	B	G	H	D	B	A	C	D	E	D	F	F	D	H	E	E
(54) C62	Clinical isolate,Male, HIV +	A/VNI	B	D	G	D	A	F	H	A	A	D	B	D	D	D	H	F	D	H	G	E
(55) C63	Clinical isolate,Male, HIV +	A/VNI	B	D	H	D	B	F	G	D	B	B	B	B	F	D	F	F	B	H	E	D
(56) C64	Clinical isolate,Male, HIV +	A/VNI	B	B	E	D	B	G	H	B	B	B	D	B	G	E	H	D	D	D	E	D
(57) C65	Clinical isolate,Female, HIV+	A/VNI	B	B	D	B	B	D	D	A	A	B	B	B	A	C	F	B	B	E	B	D
(58) C67	Clinical isolate,Male, HIV +,	A/VNI	B	B	F	B	A	B	B	A	B	A	B	B	B	A	E	B	D	D	B	B
(59) C68	Clinical isolate,Male, HIV +	A/VNI	D	B	D	D	A	G	H	B	A	A	B	A	F	D	H	E	A	F	E	D
(60) C69	Clinical isolate,Female, HIV+	A/VNI	A	D	C	B	A	G	G	A	D	A	A	B	G	G	H	D	D	H	D	B
(61) C70	Clinical isolate,Male, HIV +	A/VNI	B	B	E	D	B	G	H	B	D	B	B	B	F	D	G	E	D	F	F	D
(62) C71	Clinical isolate,Male, HIV +	A/VNI	D	B	F	E	D	H	F	D	B	B	B	A	E	F	G	E	D	G	F	D
(63) C72	Clinical isolate,Male, HIV +	A/VNI	D	E	G	F	B	H	G	B	D	D	D	A	D	F	H	B	D	G	E	D
(64) C73	Clinical isolate,Male, HIV +	A/VNI	A	D	D	D	D	G	F	B	A	B	A	B	E	D	G	D	D	F	E	D
(65) C74	Clinical isolate,Male, HIV +	A/VNI	D	D	E	D	B	F	F	B	B	B	A	B	F	E	H	D	B	F	D	D
(66) C75	Clinical isolate,Male, HIV +	A/VNI	C	D	H	D	D	G	G	B	B	D	D	D	G	F	F	E	B	H	G	B
(67) C76	Clinical isolate,Male, HIV +	A/VNI	A	A	D	D	A	G	H	A	A	B	B	D	F	E	G	B	D	H	D	B
(68) C81	Clinical isolate,Male, HIV +	A/VNI	B	D	E	D	B	E	F	B	A	D	B	B	D	D	F	B	D	D	D	D
(69) C84	Clinical isolate,Male, HIV +	A/VNI	B	B	F	F	B	H	F	B	B	B	D	D	E	G	F	E	D	G	F	E
(70) C92	Clinical isolate,Female, HIV+	A/VNI	B	D	E	D	B	G	H	B	A	D	B	B	F	D	F	C	C	G	E	E
(71) C93	Clinical isolate,Male, HIV +	A/VNI	A	D	D	D	A	G	H	D	A	D	B	B	E	D	E	B	D	H	D	D
(72) C96	Clinical isolate,Male, HIV +	A/VNI	B	D	E	D	B	F	G	B	A	D	E	B	E	B	F	D	C	G	G	E
(73) C101	Clinical isolate,Male, HIV +	A/VNI	B	D	E	D	B	F	G	A	A	D	D	B	E	B	E	B	A	H	D	D
(74) C102	Clinical isolate,Female, HIV+	A/VNI	A	D	D	D	A	G	H	A	A	D	B	C	E	D	E	B	D	H	E	E
(75) C103	Clinical isolate,Female, HIV+	A/VNI	D	D	G	F	B	H	D	A	D	D	B	A	D	D	F	D	D	E	E	F

Table 1 (*Continued*)

Species/Varieties Strain number	Characteristics	Serotype/Molecular Type	KYQU31	KYQU45	KYQU112	KYHB88	KYHB55	KYQU72	KYQU88	KYLV22	KYBI249	KYLV91	KYQU99	KYLV73	KYE002	KYLC60	KYQU139	KYLV102	KYQU127	KYQU83	KYQU100	KYLC83
C. n. var. neoformans																						
(1) ATCC28957	Human body lesion	D/VNIV	B	D	D	B	B	H	G	B	B	D	B	B	G	G	D	C	C	G	C	B
(2) ATCC28958	Pigeon droppings	D/VNIV	B	B	D	C	A	G	G	A	C	C	B	B	F	F	E	C	D	G	C	B
(3) EI0	Pigeon droppings	D/VNIV	A	D	D	C	A	G	E	A	A	C	B	B	F	F	D	B	D	G	C	C
(4) EI1	Eucalyptus spp	D/VNIV	A	D	D	D	B	F	G	A	C	C	B	B	F	E	D	C	D	G	D	C
(5) EI2	Eucalyptus spp	D/VNIV	B	C	B	C	A	G	F	B	A	D	A	C	G	F	D	C	D	G	B	D
(6) EI3	Eucalyptus spp	D/VNIV	A	D	D	C	B	G	G	A	A	C	B	B	G	E	D	B	D	G	B	B
(7) EI4	Eucalyptus spp	D/VNIV	A	D	D	C	A	G	G	A	A	C	B	B	G	F	D	B	D	G	B	B
(8) EI5	Eucalyptus spp	D/VNIV	B	C	D	B	B	F	G	A	A	C	B	A	E	F	B	B	D	F	D	C
(9) EI6	Eucalyptus spp	D/VNIV	A	D	D	C	B	G	E	A	B	C	A	C	G	E	D	C	D	F	B	B
(10) EI7	Eucalyptus spp	D/VNIV	B	C	C	C	A	G	G	B	B	C	A	B	F	E	E	B	D	H	C	C
(11) EI8	Eucalyptus spp	D/VNIV	A	D	D	D	B	G	G	A	A	C	B	C	G	F	D	C	D	G	B	C
(12) EI9	Eucalyptus spp	D/VNIV	A	D	E	B	A	G	G	A	A	C	A	B	G	E	G	B	E	G	E	C

[a]Killer yeasts: *Kodamaea ohmeri* (KYHB55, KYHB88); *Candida casmulans* (KYQU31, KYQU127, KYLV102); *Trichosporon faecale* (KYQU45, KYQU100, KYQU88, KYQU72, KYQU112); *Kluyveromyces marxianus* (KYLV22); *Trichosporon japonicum* (KYBI249, KYE002, KYQU139); *Trichosporon aoides* (KYLV91); *Kluyveromyces lactis* (KYQU99, KYQU73); *Trichosporon coremiiforme* (KYLC60, KYLC83, KYQU83).

A: no halo; B: 1–4mm; C: 4–10mm; D: 11–15mm; E: 16–20mm; F: 21–25mm; G: 26–30mm; H: >31mm.

Highlighted columns indicate that the killer yeasts with correlation between the killer sensitivity and the variety or species of *Cryptococcus* tested. A, B and C scores are highlighted in bold; D, E, F, G and H are highlighted in grey.

inhibition halo was observed by means of optical and scanning electron microscopy 15, 21 and 27 hours after inoculation.

Optical and Scanning electron microscopy (SEM) of cells within the inhibition halo

Optical microscopic observations were performed at a 1000× magnification. For the scanning electron microscopy, small blocks of the YM-MB medium containing dead cells from within the inhibition halos were collected for fixation [18]. The samples were fixed overnight at 4°C with 2% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. The specimens were rinsed in the same buffer, dehydrated in a series of 30–100% acetone solutions, dried to the critical point in CO_2 (CPD 030 BALTEC), and coated with gold in a sputter-coater (SCD 050 BALTEC). This material was examined in a Jeol JSM 5800 scanning electron microscope (SEM) at the Electron Microscopy Center of the Universidade Federal do Rio Grande do Sul (CME/UFRGS, Porto Alegre/RS). Control samples (small blocks of YM-MB medium containing a rug of intact cells from the sensitive *Cryptococcus* isolates) provided sufficient information on the structural features of undamaged cells.

Results

The identification of killer yeasts and data concerning the growth inhibition halos of the clinical and environmental isolates of *C. neoformans* and *C. gattii* are shown in Table 1. All strains were inhibited by at least eight killer yeasts, 65% were inhibited by more than 17 yeasts, 82% by more than 15 yeasts, and 14% by all killer yeasts. *T. japonicum* KYQU139 inhibited all the *Cryptococcus* isolates, while *T. faecale* KYQU45, KYQU88 and *T. coremiiforme* KYLC83 inhibited all but one. Sensitivity patterns to the killer toxins demonstrated that there is a correlation between sensitivity to strains KYQU45, KYQU112, KYQU100 and KYLC83 sensitivity and the variety or species of *Cryptococcus*. *C. neoformans* var. *grubii*, was found to be the most sensitive, with halos ≥ 11mm (letters D, E, F, G or H). In contrast, *C. gattii* was much less sensitive, with some isolates total resistant (letter A). Isolates of *C. neoformans* var. *neoformans* were found to be both resistant and intermediately sensitive. Taking into account the number of isolates for each *Cryptococcus* species or variety and the four killer yeasts capable of discriminating them (KYQU45, KYQU112, KYQU100 and KYLC83), we calculated the percentage of strains in groups A, B and C. The results were as follows; *C. gattii* (40%), *C. neoformans* var. *neoformans* (62.5%) and *C. neoformans* var. *grubii* (19%). Clearly, *C. neoformans* var. *grubii* are more sensitive than the others. If we take into account only the number of isolates with A scores, the results are the following; *C. gattii* (9.6%), *C. neoformans* var. *grubii* (2%) and *C. neoformans* var. *neoformans* with no A, showing its intermediate sensitivity (Table 1). There was no correlation between killer sensitivity and the variety, molecular type and species for all the other killer strains tested in this study. A hierarchical cluster based on the quantitative data matrix (QDM) is shown in Fig. 1, which indicates that there was complete discrimination of susceptibility among the *Cryptococcus* isolates tested by means of killer sensitivity typing.

Scanning electron microscopy of the varieties of *C. neoformans* after 24 h of action of the killer toxin revealed globular cells between 4 and 5 μm in diameter and a large amount of scattered small protusions (bud scars), without formation of pores or cell wall disruption (Fig. 2). In *C. neoformans* var. *grubii* strains, a peculiar rippled surface and sharp structures, probably buds among the G and S-phases, were observed all over the surface of the cells. The cells of *C. neoformans* var. *neoformans* just presented an undulating surface, without the presence of the sharp structures. Observations made with optical microscopy revealed that the cells of both varieties of *C. neoformans* presented an alteration in the growth pattern, when compared to controls cells. Specifically, we noted cells without buds (G1-phase cells), a significant number of cells with small buds (the diameter of the bud having less than 1/3 of parent cell diameter = S-phase cells) and a smaller number of cells with medium and large buds (the diameter of the buds being more than ½ and 2/3 of the parent cell diameter = M-phase cells). A larger number of cells in the S-phase and M-phase were observed within the inhibition halo of *C. gattii*, but this did not allow a clear differentiation of the species. Optical and SEM microscopic observations were similar after 21 and 27 hours of action of the killer toxins. Cells within the inhibition halo were streaked on YM-MB agar plates and incubated at 25°C for 24, 48, 72 and 96 h. No growth was found, suggesting that the toxins may have a fungicidal mode of action. To confirm these results, cells were also inoculated into YM broth and incubated under the same conditions. No growth was again noted for these strains. Through India ink staining we noted the presence of the polysaccharide capsule in *C. gattii* after the dehydration procedure for SEM in dead cells and cells which have not been in contact with the killer strains (result not shown).

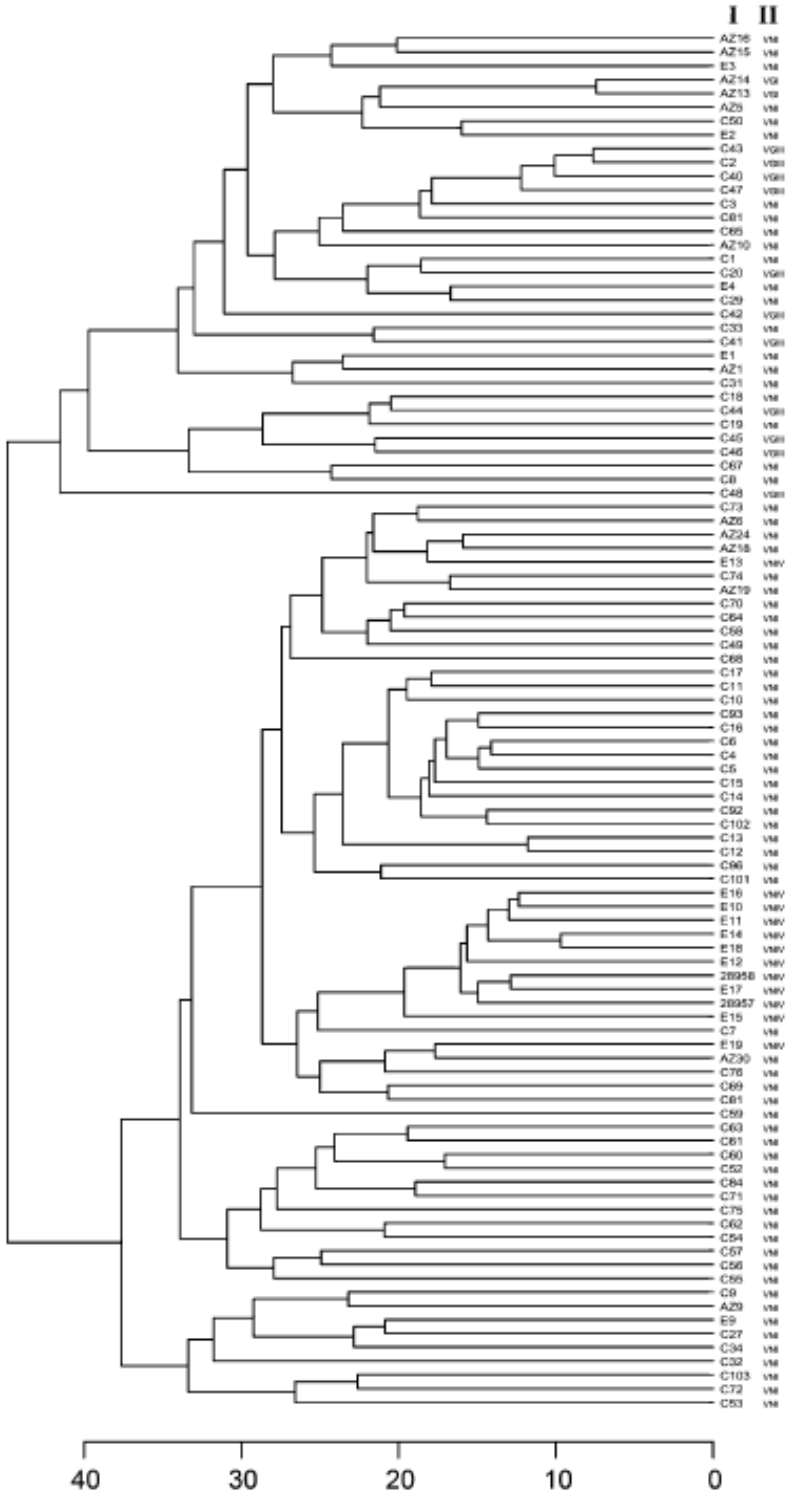

Fig. 1 Dendrogram of *Cryptococcus neoformans* and *Cryptococcus gattii* strains obtained by the killer sensitivity patterns analyzed through a quantitative data matrix (QDM). I. Strain numbers. II. Molecular type following the molecular standards provided by the Molecular Mycology Laboratory in Sydney, Australia [12].

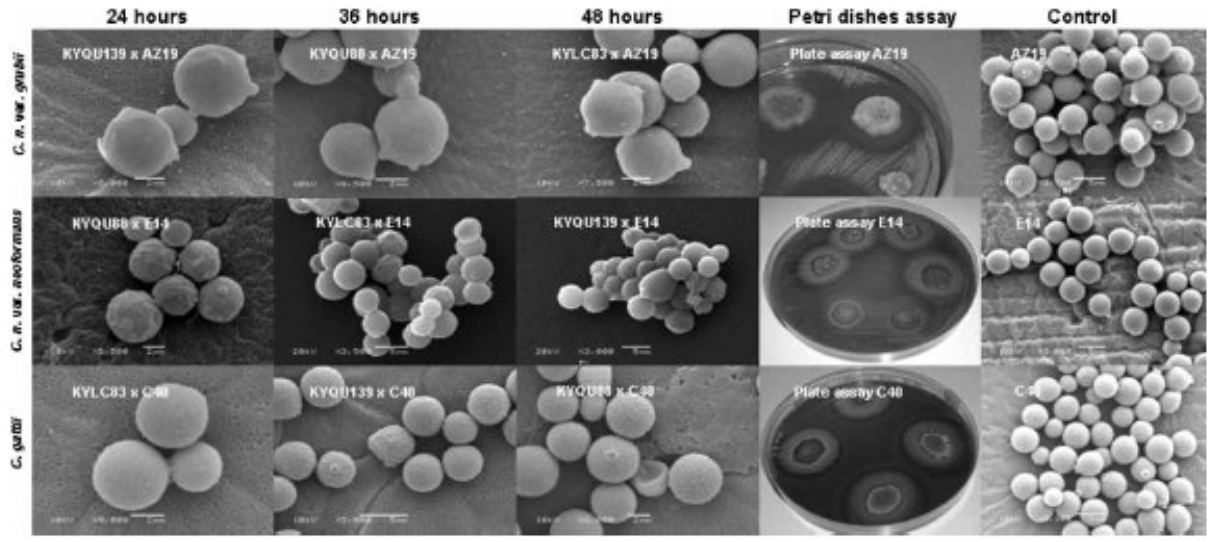

Fig. 2 Scanning electron microscopy (SEM) of *Cryptococcus neoformans* var. *grubii* (AZ19), *Cryptococcus neoformans* var. *neoformans* (E14) and *Cryptococcus gattii* (C40) exposed to the following killer yeasts: *T. japonicum* KYQU139, *T. faecale* KYQU88 and *T. coremiiforme* KYLC83. The corresponding killer plate assay and the SEM of the control alive *Cryptococcus* cells are demonstrated at the rightmost of the figure. Bar scales are presented in each image.

Discussion

The high level of inhibition of *Cryptococcus* isolates by killer yeasts is very interesting, especially in light of the fact that isolates had similar sensitivity patterns, irrespective of their clinical/environmental origins, species or variety. Five killer strains were capable of inhibiting at least 99% of the *Cryptococcus* isolates, and seem to be of promise as the source of new antifungal agents aimed at controlling this microorganism. This is crucial because prolonged use of antifungals in treating cryptococcal meningitis possesses several disadvantages due to the toxic effects of the agents and the development of resistance [19,20].

There was no correlation among the isolates obtained from different patients, hospitals, molecular types, or areas in the state of Rio Grande do Sul, Brazil (Fig. 1). Strains obtained from different hospitals had similar molecular types, making their tracking difficult. The method proposed in the present study could complement the data obtained by molecular fingerprinting, discriminating the molecular types into phenotypic groups based on their sensitivity patterns to killer toxins. This would be important in cases of nosocomal infections or in epidemiological studies. The diversity of cell-wall receptors involved in the binding of killer toxins could be the basis for the different sensitivity patterns demonstrated by strains belonging to the same molecular type, but more experiments are necessary to clarify this point.

The presence or absence of killer sensitivity allowed Boekhout and Scorzetti [9] to distinguish ten different killer types within *C. neoformans* and *C. gatti* using six killer basidiomycetous yeasts. Their smaller discriminatory power, when compared to our data, is probably due to the following three factors; low number of killer yeasts, no hierarchical cluster analysis of the binary data and the use of qualitative rather than quantitative data (halo diameter). Lemmer *et al.* [11] using Fourier transform infrared (FT-IR) spectroscopy as an additional typing method to PCR fingerprinting obtained results similar to ours with 110 isolates of *C. neoformans*. However, the QDM fingerprint method based on killer sensitivity patterns is a cheaper methodology (it does not require sophisticated equipments nor licensed softwares) and is easy to perform, appropriate for laboratories with minimal resources. It could also be applied in control laboratories for registration, patenting, recognition and quality checking of culture collections.

In this work we report an innovative technique for the analysis of susceptibility of the sensitive strains to the injurious cellular effect of the toxins secreted by the killer yeasts. This technique is based on the fact that the cells of the sensitive strain are able to begin multiplication before the toxin is produced and can act. Thus, the inhibition halo is not devoid of cells, but cells present are damaged by the action of the toxin. This indicates that death depends on the accumulation of the toxin secreted from killer strain into the media, and

this process takes some time. Several mechanisms of action have already been reported, i.e., disruption of the cytoplasmatic membrane with formation of ion channels and release of ATP, blockage of the DNA synthesis, and cell cycle arrest at the G1/S phases [21]. Optical and SEM microscopy of cells within the inhibition halo could provide the basis for clarifying the mechanism of action of these toxins. The high number of cells with small buds or without buds in *C. neoformans* isolates exposed to the toxins, allied to the absence of visible pores or extrusion of the cytosol, suggest that these toxins act by interrupting cell cycle development. The larger number of cells in the S and M-phase within the inhibition halo of *C. gattii* suggest that cell cycle arrest may happen in a different phase in this species, but this has to be tested. The mucoid substance in *C. gattii* strains, possibly the more abundant polysaccharide capsule in comparison to *C. neoformans*, might have been responsible for the non perception of morphologic alterations in their cellular wall by SEM.

Vadasz *et al*. [22], evaluating the effect of the K2 toxin on cells of *S. cerevisiae* by SEM observed the extrusion of the cellular cytosol. However, the authors used mixed populations of sensitive and killer yeasts and observed the results in the stationary growth phase, when other factors may be influencing cell death. Knowing that the process of toxin secretion and accumulation in the media might be highly variable between different laboratories, the use of purified toxins should reduces those differences. But the diversity of cell-wall receptors involving in the binding of killer toxins is probably the basis for the different sensitivity patterns demonstrated by *Cryptococcus* strains.

In conclusion, the *Cryptococcus* strains analysed had a pattern of high susceptibility to killer toxins. We were able to select some killer yeasts that produce toxins which show promised for the development of new antifungal agents aimed at the treatment of cryptococosis. There was a complete discrimination of the strains using a typing method based on killer sensitivity patterns. This method cannot replace genotyping, but killer sensitivity typing and PCR-fingerprinting can be used together to enhance the discrimination of isolates in epidemiological studies. Optical and SEM microscopy showed that there was no formation of pores on the cell surface of the sensitive cells in contact with the toxins. This might suggest that the killing mechanism does not act through the release of cellular components through pores produced on the cell surface. However, to clearly establish the action of the toxin more studies are necessary.

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References

1 Lin X, Heitman J. The biology of the *Cryptococcus neoformans* species complex. *Annu Rev Microbiol* 2006; **60**: 69–105.
2 Levitz SM. The ecology of *Cryptococcus neoformans* and the epidemiology of cryptococcosis. *Rev Infect Dis* 1991; **13**: 1163–1169.
3 Kwon-Chung KJ, Boekhout T, Fell JW, *et al*. Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacillisporus* (Basidiomycota, Hymenomycetes, Tremellomycetidae). *Taxon* 2002; **51**: 804–806.
4 Franzot SP, Salkin IF, Casadevall A. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. *J Clin Microbiol* 1999; **37**: 838–840.
5 Meyer W, Castañeda A, Jackson S, *et al*. Molecular typing of iberoamerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis* 2003; **9**: 189–195.
6 Boekhout T, Theelen B, Diaz M, *et al*. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiol* 2001; **147**: 891–907.
7 Diaz MR, Boekhout T, Theelen B, *et al*. Molecular sequence analyses of the intergenic spacer (IGS) associated with rDNA of the two varieties of the pathogenic yeast, *Cryptococcus neoformans*. *Syst Appl Microbiol* 2000; **23**: 535–545.
8 Diaz MR, Boekhout T, Kiesling T, *et al*. Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast *Cryptococcus neoformans*. *FEMS Yeast Res* 2005; **5**: 1129–1140.
9 Boekhout T, Scorzetti G. Differential killer toxin sensitivity patterns of varieties of *Cryptococcus neoformans*. *J Med Vet Mycol* 1997; **35**: 147–149.
10 Farina C, Arosio M, Moioli F, *et al*. Phenotyping of *Cryptococcus neoformans* strains in Bergamo, Italy. *New Microbiol ; 26* 1985; **2003**: 83–90.
11 Lemmer K, Naumann D, Raddatz B, *et al*. Molecular typing of *Cryptococcus neoformans* by PCR fingerprinting in comparison to serotyping and Fourier Transform Infrared Spectroscopy used for phenotyping. *Med Mycol* 2004; **42**: 135–147.
12 Casali A, Goulart L, Rosa e Silva LK, *et al*. Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *FEMS Yeast Res* 2003; **3**: 405–415.
13 Abegg M, Cella F, Faganello J, *et al*. *Cryptococcus neoformans* and *Cryptococcus gattii* isolated from the excreta of Psittaciformes in a southern brazilian zoological garden. *Mycopathologia* 2006; **161**: 83–91.
14 O'Donnell K. *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds). *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*. CAB International, Wallingford, UK, 1993: 225–233.

15 Altschul SF, Madden TL, Schäffer AA, *et al*. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; 25: 3389–3402.
16 Fuentefria A.M., Franskoviaki I., Mercado L., *et al*. Inhibition of clinical and environmental *Cryptococcus neoformans* isolates by two Brazilian killer yeasts. *J Basic Microbiol* 2006; 2: 87–93.
17 Buzzini P, Berardinelli S, Turchetti B, *et al*. Fingerprint of yeasts at the strains level by differential sensitivity responses to a panel of selected killer toxins. *System Appl Microbiol* 2003; 26: 466–470.
18 Faganello J, Arruda W, Schrank A, *et al*. An alternative method to prepare samples of the pathogenic yeast *Cryptococcus neoformans* for scanning electron microscopy analysis. *J Microbiol Meth* 2006; 64: 416–419.
19 Maxwell MJ, Messer SA, Hollis RJ, *et al*. Evaluation of E-test method for determining voriconazole and amphotericin B MICs for 162 clinical isolates of *Cryptococcus neoformans*. *J Clin Microbiol* 2003; 41: 97–99.
20 Viviani MA. Cryptococcal meningitis: diagnosis and treatment. *Int J Antimicrob Agents* 1996; 6: 169–173.
21 Magliani W, Conti S, Gerloni M, *et al*. Yeast killer systems. *Clin Microbiol Rev* 1997; 10: 369–400.
22 Vadasz AS, Jagganath DB, Pretorius IS, *et al*. Electron microscopy of the K2 killer effect of *Saccharomyces cerevisiae* T206 on a mesophilic wine yeast. *Antonie van Leeuwenhoek* 2000; 78: 117–122.

ANEXO 4

ABEGG, M. A.; CELLA, F. L.; FAGANELLO, J.; VALENTE, P.; SCHRANK, A. & VAINSTEIN, M. H. *Cryptococcus neoformans* and *Cryptococcus gattii* isolated from the excreta of Psittaciformes in a Southern Brazilian Zoological Garden. *Mycopathologia*, 161: 83–91, 2006.

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Cryptococcus neoformans and *Cryptococcus gattii* isolated from the excreta of Psittaciformes in a Southern Brazilian Zoological Garden

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Abstract

Cryptococcus neoformans, a major pathogen in immunocompromised patients, is a ubiquitous free-living fungus that can be isolated from soils, avian excreta and plant material. To further study potential saprophytic sources of this yeast in the Southern Brazilian State Rio Grande do Sul, we analyzed fecal samples from 59 species of captive birds kept in cages at a local Zoological Garden, belonging to 12 different orders. Thirty-eight environmental isolates of *C. neoformans* were obtained only from Psittaciformes (Psittacidae, Cacatuidae and Psittacula). Their variety and serotype were determined, and the genetic structure of the isolates was analyzed by use of the simple repetitive microsatellite specific primer M13 and the minisatellite specific primer $(GACA)_4$ as single primers in the PCR. The varieties were confirmed by pulsed-field gel electrophoresis (PFGE). Thirty-three isolates (87%) were from the var. *grubii*, serotype A, molecular type VNI and five (13%) were *Cryptococcus gattii*, serotype B, molecular type VGI. All the isolates were mating type α. Isolates were screened for some potential virulence factors. Quantitative urease production by the environmental isolates belonging to the *C. gattii* was similar to the values usually obtained for clinical ones.

Key words: avian excreta, cryptococcosis, *Cryptococcus gattii*, *Cryptococcus neoformans*, molecular typing, virulence factors

Introduction

Cryptococcus neoformans, a basidiomycetous yeast, is the etiologic agent for cryptococcosis, a major opportunistic mycosis in patients with AIDS [1–3]. Strains of *C. neoformans* are traditionally divided into five distinct serotypes: A, B, C, D and AD [1]. Due to the fact that strains of different serotypes often exhibit significant divergence at molecular level, three biochemically and genetically distinct varieties have been proposed to accommodate this divergence: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *gattii* (serotypes B and C). *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* correspond to the teleomorph *Filobasidiella neoformans* var. *neoformans* and *C. neoformans* var. *gattii* to the teleomorph *F. neoformans* var. *bacillispora* [1, 3–5]. However, sequence analysis of the intergenic spacer (IGS) associated with rDNA [6] and amplified fragment length polymorphism (AFLP) genotyping [7] revealed significant differences in nucleotide composition and a considerable genetic divergence between and

within the former three varieties proposed, indicating that *C. neoformans* var. *grubii* (serotype A) should not be recognized as a separate variety [6]. Based on these studies the authors proposed to divide *C. neoformans* into two separate species, with serotypes A, D, and AD included in the species *C. neoformans* (Sanfelice) Vuillemin, and strains of serotype B and C constituting a new species, *Cryptococcus bacillisporus* Kwon-Chung [6, 7]. More recently a proposal to conserve the name *Cryptococcus gattii* against *C. bacillisporus* was made [8].

The incidence of cryptococcosis caused by *C. neoformans* var. *grubii*/*neoformans* has been increasing steadily because of the AIDS epidemic and the growing use of immunossupressive drugs. Pigeon (*Columba livia*) excreta are considered to be the principal source of these varieties, which explains why the majority of studies have been directed at investigating the fungus in this substrate [1, 9, 10]. However, the frequent isolation of the vars. *grubii* and *neoformans* from excreta of other birds, such as psittacines in zoos, pet shops and private households [11–14], the evidence of zoonotic transmission of the fungus to an immunocompromised patient [15], and some other evidences [16–18], points to the excreta of these birds as possible important environmental reservoirs of this pathogen. In Brazil, *C. gattii* occurs associated with *Eucalyptus* spp. and some other trees and the infection is endemic, especially in the north and northeast of the country being 62.7% of the clinical isolates and 62% of the environmental isolates from serotype B [19]. *C. gattii* was previously associated with deaths of psittacine birds in a breeding aviary in São Paulo, Brazil, and it was also isolated from excreta of these birds. Histological examination of the birds showed cryptococcal cells in the beak, choana, sinus, lungs, air sacs, heart, liver, spleen, kidneys, intestines and central nervous system [14]. In the present report the birds were healthy and did not present incoordination, progressive paralysis, difficulty in flying or superficial lesions.

PCR fingerprinting and other molecular techniques have extended our knowledge of the epidemiology of cryptococcosis over the last few years. PCR fingerprinting has been used to amplify hypervariable repetitive DNA sequences in *C. neoformans*. The pattern generated with the oligonucleotide primer 5′(GACA)$_4$-3′ and the microsatellite specific primer M13 are able to classify *C. neoformans* strains in serotypes A, B/C, or D, showing the sensitivity to detect both inter- and intravarietal differences [9, 10, 20–25]. PCR for *MAT*α and *MAT*a pheromones is being used to determine mating type, ploidy, and variety [26]. Electrophoretic karyotyping (EK) has also been shown to be a useful technique for distinguishing *C. neoformans* isolates. The general differences in chromosome sizes and numbers allow a reasonable prediction of the strain's variety status from its initial karyotype pattern [1, 27, 28].

The present study was motivated by the occurrence of cryptococcosis in the Southern Brazilian State Rio Grande do Sul, and by the fact that excreta of birds other than pigeons have never been analyzed as potential natural reservoirs and sources of the fungus in this state. Our purposes were: (1) verify the presence of *C. neoformans* in the excreta obtained from captive birds at a local Zoo; (2) screen some virulence-associated traits among the isolates; (3) survey the presence of the mating types alleles: α and a; (4) apply (GACA)$_4$, M13 PCR fingerprinting and EK to identify the varieties.

Materials and methods

Environmental and animals sampling

Desiccated excreta from 55 cages with 59 species of birds belonging to 12 different orders (Psittaciformes, Piciformes, Galliformes, Falconiformes, Casuariiformes, Strigiformes, Ciconiiformes, Anseriformes, Rheiformes, Struthioniformes, Passeriformes and Columbiformes) were collected in the summer at the Zoological Garden of Sapucaia do Sul, state of Rio Grande do Sul, Brazil, and examined for the presence of *C. neoformans*. The majority of the birds were kept as the only species per cage apart from two of these cages each one with two and three species of birds. Imported or quarantine animals were never considered. The examined birds were healthy with a residency of at least 3 months, and none of them had been treated with drugs for at least 1 week before sampling. Air contamination was verified with birdseed agar plates supplemented with ampicillin (150 μg ml^{-1}), chloramphenicol (150 μg ml^{-1}) and biphenyl

Table 1. C. neoformans isolates from Psittaciformes studied by environmental sources, variety (serotypes) and molecular type as identified by PCR fingerprinting with the primers $(GACA)_4$ and M13

Bird scientific name (common name)	Isolate	Identification	Serotype	Molecular type
Aratinga leucophthalmus (White-eyed parakeet)	AZ01; AZ02; AZ03	*C. n.* var. *grubii*	A	VNI
Pionus maximiliani (Scaly-headed parrot)	AZ04; AZ05; AZ06; AZ07	*C. n.* var. *grubii*	A	VNI
Nymphicus hollandicus (Cockatiel)	AZ 08	*C. n.* var. *grubii*	A	VNI
Psittacula eupatria (Alexandrine parakeet)	AZ15; AZ16; AZ17	*C. n.* var. *grubii*	A	VNI
Nandayus nenday (Nanday parakeet)	AZ18; AZ19; AZ20; AZ21; AZ22; AZ23	*C. n.* var. *grubii*	A	VNI
Aratinga aurea (Peach-fronted conure)	AZ24; AZ25; AZ28	*C. n.* var. *grubii*	A	VNI
Aratinga aurea (Peach-fronted conure)	AZ26; AZ27	*C. gattii*	B	VGI
Aratinga jandaya (Jandaya parakeet)	AZ09; AZ10; AZ11	*C. n.* var. *grubii*	A	VNI
Amazona festiva (Festive parrot)	AZ29; AZ30; AZ31; AZ33; AZ34; AZ35; AZ36; AZ37	*C. n.* var. *grubii*	A	VNI
Amazona festiva (Festive parrot)	AZ32	*C. gattii*	B	VGI
Amazona rhodocorytha (Red-brown amazon parrot)	AZ12	*C. n.* var. *grubii*	A	VNI
Amazona farinosa (Mealy parrot)	AZ13; AZ14	*C. gattii*	B	VGI
Amazona farinosa (Mealy parrot)	AZ38	*C. n.* var. *grubii*	A	VNI

(0.1%) [9], which where left open during 15 min inside the cages and also outside by 0.5 m of the cages. These plates where incubated for 14 days a 30 °C with daily observation. Samples from hollows of two eucalypt trees situated near the cages were also collected as previously described [4].

Fecal specimens sampling and processing

Approximately 20 g of excreta were taken from the cage floors using sterile spatulas, put into sterile plastic containers and processed on the same day. Approximately 1 g of the avian excreta was diluted in 9 ml of sterile saline solution (0.9% NaCl), vortexed for 10 min, and allowed to settle for 1 h. Aliquots of 10^{-2} and 10^{-3} dilutions were inoculated in duplicate onto birdseed agar plates supplemented with ampicillin (150 μg ml^{-1}), chloramphenicol (150 μg ml^{-1}) and biphenyl (0.1%) [9]. The plates were incubated at 30 °C and examined each day for the presence of colonies showing the brown color effect (BCE). The colonies with BCE were enumerated after 72–96 h of growth and the plates were observed for a period up to 14 days. Representative BCE colonies were streaked twice in birdseed agar, and the isolates were maintained in Sabouraud-dextrose agar slants until identification and characterization [9].

Identification and maintenance of the isolates

The identification of the isolates included microscopic analysis of India ink preparations, thermotolerance at 37 °C in Sabouraud dextrose agar, cycloheximide sensitivity (0.1%), qualitative urease activity [29], NaCl sensitivity, nitrate and carbon assimilation tests [9, 30]. The canavanine-glycine-bromothymol blue (CGB) medium was used to determine the variety of the isolates [31]. Serotyping was performed using the Crypto Check kit (Iatron Labs, Tokyo, Japan) according to the manufacturer's instructions. After identification,

the *C. neoformans* isolates were maintained on Sabouraud dextrose agar at 4 °C and in glycerol stocks at −20 °C.

Assay of virulence factors

Extracellular phospholipase production was assayed in Sabouraud dextrose agar with 1 M sodium chloride, 5 mM calcium chloride and 8% sterile egg yolk [10]. The diameter of the zone of precipitation around the colonies was measured after 7 days incubation at 30 °C. The index of phospholipase activity (P_z) was a ratio between the colony diameter and the total diameter of the colony plus precipitation zone. A P_z value of 1.0 indicated that the test sample was phospholipase negative. Urease activity was determined with a phenol–hypochlorite reaction, according to Weatherburn [32].

Determination of mating type by PCR

DNA was extracted as previously described [10]. Two PCR primer pairs, specific for mating type α and a, were used, according to Chaturvedi et al. [26]. The α-mating-type-specific 5′ -primer was 5′-CTTCACTGCCATCTTCACCA-3′ (*MAT*α1) and the 3′ -primer was 5′-GACACAAAGGGTCA TGCCA-3′ (*MAT*α2). The a-mating-type-specific 5′-primer was 5′-CGCCTTCACTGCTACCTTC T-3′ (*MAT*a1) and the 3′ -primer was 5′-AACGCA AGAGTAAGTCGGGC-3′ (*M AT*a2). Amplification reactions were performed in a volume of 25 μl. The PCR amplicons were electrophoresed on 2% agarose gels in 1× Tris-borate-EDTA (TBE) buffer at 80 V for 90 min and then stained in a solution of 0.5 μg ml^{-1} ethidium bromide.

PCR fingerprinting

Oligonucleotides of the minisatellite specific core sequence of the wild-type phage M13 (5′-GAG-GGTGGCGGTTCT-3′) and of the microsatellite specific sequence $(GACA)_4$ were used as single primers in the PCR [21]. Amplification products were separated by electrophoresis in 1.4% agarose gels in 1× TBE buffer stained with ethidium bromide at 0.5 μg ml^{-1} for 7 h at 60 V and visualized under UV light. PCR fingerprinting types (VNI–VNIV and VGI–VGIV) were assigned according to the molecular standards provided by the Molecular Mycology Laboratory in Sydney, Australia.

Electrophoretic karyotyping

Karyotype analysis was done by contour-clamped homogeneous electrophoresis (CHEF DR-II). *C. neoformans* chromosomal DNA plugs were prepared using a modification of existing protocols [27, 28]. For EK, the plugs were inserted into a 1% pulsed-field-certified agarose (Bio-Rad) gel (12 by 14 cm), and electrophoresis was performed in a CHEF DR-II variable-angle pulsed-field electrophoresis system (Bio-Rad) in 0.5× TBE buffer (1× TBE is 89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA pH 8.3) at 14 °C. Electrophoretic conditions were as follows: pulse intervals of 60 to 120 s for 39 h at 6 V/cm. The gels were stained with ethidium bromide and photographed. A *Saccharomyces cerevisiae* chromosomal DNA size standard was inserted in each gel as a molecular weight standard.

Results

Isolation and identification of C. neoformans

C. neoformans was isolated from 10 out of 55 bird excreta samples (18.18%). Positive samples were only from excreta of psittacine birds maintained as one species per cage located in the same area. Thirty-eight isolates were obtained. The positive psittacines fecal samples were further screened during the winter but isolates of *C. neoformans* were not obtained. Microscopic examination of fresh isolates in birdseed agar with India ink preparations was used to observe the presence of capsule in the isolates. Thirty-three out of 38 isolates exhibited non-capsulated cells or cells with a tinny capsule, associated with smooth and dry colony morphologies on birdseed agar. Five isolates presented medium to large capsules, correlating with a mucous and brilliant colony morphologies on the same medium. All isolates were nitrate-negative, urease-positive and their growth was inhibited in presence of cycloheximide 0.1% and NaCl 16%. All isolates were *C. neoformans* according to the assimilation pattern of

19 carbon compounds. Glucose, galactose, sorbose, sucrose, trehalose, raffinose, maltose, D-ribose, L-rhamnose, D-xylose, myo-inositol and D-mannitol were assimilated by all isolates, whereas lactose, melibiose, *N*-acetil-glucosamine, L-arabinose and glycerol were not. Assimilation of cellobiose, and glucosamine was variable. Thirty-three isolates (87%) were *C. neoformans* var. *grubii*/*neoformans* according to biotyping in CGB agar and 5 (13%), as *C. gattii*. Serotypes were confirmed with commercial monoclonal antibodies (Iatron Labs, Tokyo, Japan). Air sampling around the cages and samples collected from eucalypt trees were negative for the presence of *C. neoformans*.

Putative virulence factors

Extracellular phospholipase production was readily observed as distinct, cream zones of precipitation around the colonies in 19 (50%) out of 38 isolates. The remaining 19 isolates failed to induce a precipitate after 7 days of incubation at 30 °C. The environmental isolates exhibited P_z values within a range of 0.69–0.94, with an average of 0.83 (data not shown). Only one out of five isolates of *C. gattii* presented phospholipase activity. The quantitative urease results are shown at Figure 1. The five isolates identified as *C. gattii* had a higher urease activity than the other isolates.

Determination of mating type by PCR

Two type strains, ATCC 28957 (serotype D, *MAT*α) and ATCC 28958 (serotype D, *MAT*a) and one reference strain (ATCC 32045 *MAT*α/a) [10] were used as positive control. A 101-bp *MAT*α fragment and a 117-bp *MAT*a fragment were amplified from the corresponding controls. All the isolates belonged to mating type α (data not shown).

PCR Fingerprinting and Electrophoretic karyotyping

The microsatellite specific primer M13 and the minisatellite specific primer $(GACA)_4$ were used to amplify DNA polymorphisms within the genome of the 38 isolates. Representative gels can be seen in Figure 2. PCR-fingerprinting with the primers $(GACA)_4$ and M13 separated the *C. neoformans* isolates into two molecular types: VNI representing serotype A, and VGI representing *C. gattii*, serotype B. The most common molecular type was VNI (serotype A, var. *grubii*), which was present in 33 (87%) isolates. Five (13%) isolates belonged to VGI (serotype B, *C. gattii*).

Data regarding EK on environmental samples of *C. neoformans* are limited. Among the three representative isolates from *C. neoformans* var. *grubii* and from *C. gattii* used for EK, four unique profiles were noted upon visual inspection by using a one band difference as the discrimination criteria (Figure 3). *C. gattii* isolates had indistinguishable patterns showing small chromosomes (less than 700 kb) seen frequently in this specie [33]. However, all *C. neoformans* var. *grubii* isolates had different karyotypes as also observed by Perfect et al. [34] for environmental isolates found in soil or bird guano in North Carolina and Ohio.

Discussion

The isolation of *C. neoformans* in nature took place for the first time from peach juice in 1895, and since the early 1950s, researchers have known that this fungus is associated with avian excreta [1, 35]. Comparative studies have suggested that excreta of some species of birds may be more likely to be contaminated with the fungus than others, being a good nutrient for growth and survival for this pathogen, and may therefore, represent a reservoir and possible source of infection [1, 17]. Although in birds clinical cryptococcosis is rare, an outbreak was described for psittacine in the families Loriidae and Psittacidae in Brazil. The birds died of disseminated cryptococcosis caused by *C. gattii* [14].

It has been difficult to link human infection to bird excreta exposure; however there are some studies that strongly suggest zoonotic transmission [15, 18]. *C. neoformans* has been already isolated from excreta of captive birds in zoos [11, 16, 36, 37] and the control of the habitats of the fungus in zoological gardens and similar establishments is considered a necessity to prevent exposure of susceptible persons [12].

The 38 isolates obtained in this work from excreta of captive birds were all identified as *C. neoformans*, with 33 identified as *C. neoformans* var. *grubii* and 5 isolates as *C. gattii*. These five isolates were confirmed as *C. gattii* by the characteristic

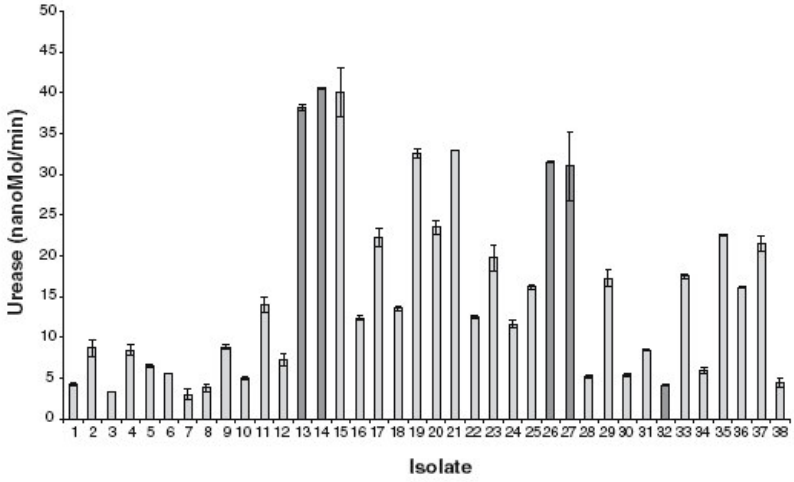

Figure 1. Urease activity of environmental samples of *C. neoformans* var. *grubii* (light shading) and *C. gattii* (dark shading) obtained from the Zoological garden. For other isolate characteristics see Table 1.

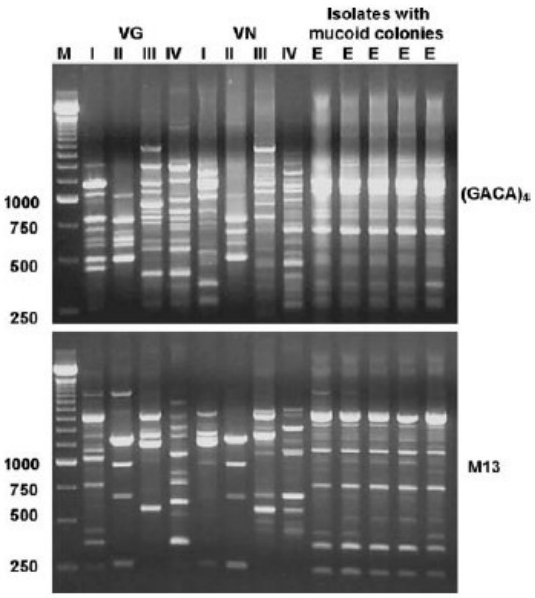

Figure 2. Representative gels of PCR fingerprinting patterns from some of the environmental *C. neoformans* isolates obtained from the Zoological garden. Upper panel primer $(GACA)_4$ and lower panel primer M13. VG and VN molecular types patterns of reference samples. M: molecular size marker, 250 bp ladder. E: environmental samples, AZ13, AZ14, AZ26, AZ27 and AZ32 respectively, all *C. gattii*. For other isolate characteristics see Table 1. Reference strains are: VGI, WM179; VGII, WM178; VGIII, WM161; VGIV, WM779; VNI, WM148, VNII, WM626; VNIII, WM628; VNIV, WM629.

color at the CGB medium, serology (serotype B), $(GACA)_4$ and M13 PCR-fingerprinting (molecular type VGI), and EK. The presence of large capsules and mucoid/brilliant colonies in the birdseed agar corresponds to the characteristic morphology of *C. gattii* [1]. *C. neoformans* was not recovered from eucalypts trees near the cages or from the birdseed agar plates left open inside the cages ruling out the possibility that the fungus was in the overall environmental rather than specifically in the bird's excreta. Reports of cryptococcosis by *C. gattii* in Brazil are sparse. The association of this variety with alternative ecological niches opens the possibility that the cryptococcosis caused by serotype B isolates in the South region of Brazil may be greater than previously thought [19].

The majority of the 38 isolates were serotype A and molecular type VNI, what is consistent with the fact that *C. neoformans* var. *grubii* is prevalent among environmental sources [20, 21]. The isolates were all obtained from excreta of psitaccines, and this material is reported in the literature as a good nutrient for *C. neoformans* growth [1]. However, the incidence of cryptococcal infection due to exposure to pet birds, such as psittacines, is unknown [1, 17]. Recently, Lagrou et al. [18] reported a case of cryptococcal meningitis in an immunocompetent patient with exposure to a pet magpie (Corvidae).

All 38 isolates analyzed were mating type α which is in accordance with the reports that

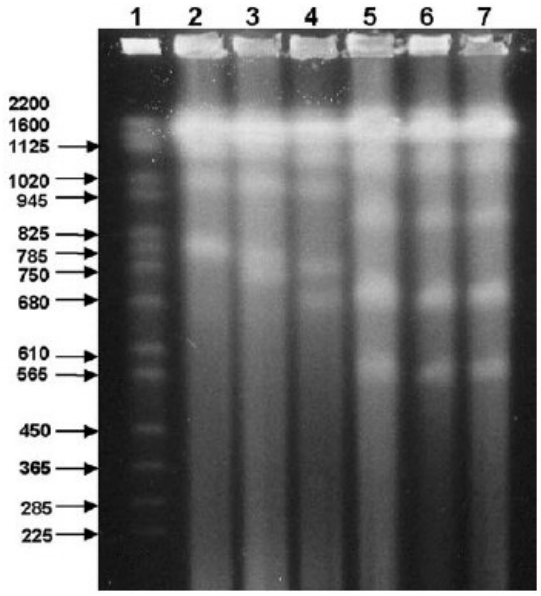

Figure 3. Electrophoretic karyotypes of environmental samples of *C. neoformans* var. *grubii* and *C. gattii* obtained from the Zoological garden. Lanes are: 1, *S. cerevisiae* DNA markers; 2, 3, and 4 correspond to samples AZ06, AZ10 and AZ12 respectively, all var. *grubii*; 5, 6 and 7 correspond to samples AZ13, AZ26 and AZ32 respectively, all *C. gattii*. Numbers in the left indicate molecular sizes in Kb. For other isolate characteristics see Table 1.

mating type α strains are 30- to 40-fold more prevalent than those of the *MAT*a among clinical as well as environmental isolates [10].

In spite of the very similar mean pH of positive and negative samples (7.3 and 7.6, respectively), pH cannot be ruled out as one of the factors that could influence *C. neoformans* survival in the fecal samples. In general, birds with a diet based on citric fruits had excreta with acidic pH (4.0–5.0), and birds with a meat-based diet had excreta with alkaline pH (8.0–9.0). As already known that alkaline pH inhibits fungal growth, this could partially explain the absence of this fungus in the excreta of some bird species.

Analysis of some putative virulence factors of the environmental isolates may suggest the infection potential of these strains. Phospholipase activity was detected in 50% of the isolates, and its P_z value is in accordance with earlier findings for environmental strains [38, 39]. Clinical isolates usually have a higher phospholipase activity, what is known to be important for the virulence of the pathogen [40]. Phospholipase P_z value was similar among the excreta belonging to both varieties. Urease activity was higher in the isolates belonging to *C. gattii* and was comparable to previous results obtained for clinical isolates in our laboratory [10]. The importance of urease activity for the pathogenesis of the fungus is still under debate, but mice infected with urease-negative mutants of *C. neoformans* had a higher percentage of survival than those infected with urease-positive controls [41].

The *C. neoformans* var. *grubii* isolates were all from molecular type VNI, which is the most prevalent worldwide among clinical and environmental strains [10, 20, 21]. The *C. gattii* strains belonged to molecular type VGI. Casali et al. [10] reported that all *C. gattii* analyzed from clinical samples of the state of Rio Grande do Sul were molecular type VGIII. However, the number of clinical isolates of this variety genotyped in that study is small and possibly non-representative of the bulk of isolates. Thus, it is not possible to affirm that the molecular type VGI will not infect people in our local environment. As such, isolation of this molecular type with an indistinguishable karyotyping suggesting the occurrence of a clonal event in the environmental should raise some concerns. Moreover, this clonal event is even more particularly important given the ongoing outbreak in Vancouver Island, BC, Canada [25]. It is clear that there is much to learn about the ecology and epidemiology of this primary pathogen and the importance of continued surveillance in the environment not only in Brazil but worldwide.

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References

1. Casadevall A, Perfect JR. *Cryptococcus neoformans*. Washington: ASM Press, 1998 541.
2. Mitchell TG, Perfect JR. Cryptococcosis in the era of AIDS – 100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev 1995; 8: 515–548.
3. Franzot SP, Salkin IF, Casadevall A. *Cryptococcus neoformans* var. *grubii*: Separate varietal status for *Cryptococcus neoformans* serotype A isolates. J Clin Microbiol 1999; 37: 838–840.
4. Ellis DH, Pfeiffer TC. Natural habitat of *Cryptococcus neoformans* var. *gattii*. J Clin Microbiol 1990; 28: 1642–1644.
5. Sorrell TC. *Cryptococcus neoformans* variety *gattii*. Med Mycol 2001; 39: 155–168.
6. Diaz MR, Boekhout T, Theelen B, Fell JW. Molecular sequence analyses of the intergenic spacer (IGS) associated with rDNA of the two varieties of the pathogenic yeast, *Cryptococcus neoformans*. Syst Appl Microbiol 2000; 23: 535–545.
7. Boekhout T, Theelen B, Diaz M, Fell JW, Hop WC, Abeln EC, Dromer F, Meyer W. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiology 2001; 147: 891–907.
8. Kwon-Chung KJ, Boekhout T, Fell JW, Diaz M. Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacillisporus* (*Basidiomycota*, *Hymenomycetes*, *Tremellomycetidae*). Taxon 2002; 51: 804–806.
9. Horta JA, Staats CC, Casali AK, Ribeiro AM, Schrank IS, Schrank A, Vainstein MH. Epidemiological aspects of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. Med Mycol 2002; 40: 1–7.
10. Casali AK, Goulart L, Rosa e Silva LK, Ribeiro AM, Amaral AA, Alves SH, Schrank A, Meyer W, Vainstein MH. Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. FEMS Yeast Res 2003; 3: 405–415.
11. Caicedo LD, Alvarez MI, Delgado M, Cardenas A. *Cryptococcus neoformans* in bird excreta in the city zoo of Cali, Colombia. Mycopathologia 1999; 147: 121–124.
12. Staib F, Schultz-Dieterich J. *Cryptococcus neoformans* in fecal matter of birds kept in cages – control of *C. neoformans* habitats. Zentralbl Bakteriol Mikrobiol Hyg 1984; 179: 179–186.
13. Criseo G, Bolignano MS, De Leo F, Staib F. Evidence of canary droppings as important reservoir of *Cryptococcus neoformans*. Zbl Bakt 1995; 282: 244–254.
14. Raso TF, Werther K, Miranda ET, Mendes-Giannini MJ. Cryptococcosis outbreak in psittacine birds in Brazil. Med Mycol 2004; 42: 355–362.
15. Nosachuk JD, Shoham S, Fries BC, Shapiro DS, Levitz SM, Casadevall A. Evidence of zoonotic transmission of *Cryptococcus neoformans* from a pet cockatoo to an immunocompromised patient. Annals Int Med 2000; 132: 205–208.
16. Staib F. Sampling and isolation of *Cryptococcus neoformans* from indoor air with the aid of the Reuter Centrifugal Sampler and *Guizotia abyssinica* creatinine agar. Zentralbl Bakteriol Mikrobiol Hyg 1985; 180: 567–575.
17. Filiú WFO, Wanke B, Agüena SM, Vilela VO, Macedo RCL, Lazéra M. Avian habitats as sources of *Cryptococcus neoformans* in the city of Campo Grande, Mato Grosso do Sul, Brazil. Rev Soc Bras Med Trop 2002; 35: 591–595.
18. Lagrou K, Eldere VAN J, Keuleers S, Hagen F, Merckx R, Verhaegen J, Peetermans WE, Boekhout T. Zoonotic transmission of *Cryptococcus neoformans* from a magpie to an immunocompetent patient. J Intern Med 2005; 257: 385–388.
19. Nishikawa MM, Lazéra MS, Barbosa GG, Trilles L, Wanke B et al. Serotyping of 467 *Cryptococcus neoformans* isolates from clinical and environmental sources in Brazil: Analysis of host and regional patterns. J Clin Microbiol 2003; 41: 73–77.
20. Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E and The Iberoamerican Cryptococcal Study Group. Molecular typing of Iberoamerican *Cryptococcus neoformans* isolates. Emerg Infect Dis 2003; 9:189–195.
21. Meyer W, Marszewska K, Amirmostofian M, Sorrell TC 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.
22. Meyer W, Mitchell TG. Polymerase chain reaction fingerprinting in fungi using single primers specific to minisatellites and simple repetitive DNA sequences: strain variation in *Cryptococcus neoformans*. Electrophoresis 1995; 16: 1648–1656.
23. Igreja RP, Lazera MS, Wanke B, Galhardo MC, Kidd SE, Meyer W. Molecular epidemiology of *Cryptococcus neoformans* isolates from AIDS patients of the Brazilian city, Rio de Janeiro. Med Mycol 2004; 42: 229–238.
24. Katsu M, Kidd S, Ando A, Moretti-Branchini ML, Mikami Y, Nishimura K, Meyer W. The internal transcribed spacers and 5.8S rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* species complex. FEMS Yeast Res 2004; 4: 377–388.
25. Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, MacDougall L, Boekhout T, Kwon-Chung KJ, Meyer W. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc Natl Acad Sci USA 2004; 101: 17258–17623.
26. Chaturvedi S, Rodeghier B, Fan J, McClelland CM, Wickes BL, Chaturvedi V. Direct PCR of *Cryptococcus neoformans MAT*α and *MAT*a Pheromones to determine mating type, ploidy, and variety: a tool for epidemiological and molecular pathogenesis studies. J Clin Microbiol 2000; 38: 2007–2009.
27. Franzot SP, Hamdan JS, Currie BP, Casadevall A. Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: Evidence for both local genetic differences and a global clonal population structure. J Clin Microbiol 1997; 35: 2243–2251.

28. Calvo BM, Colombo AL, Fischman O, Santiago A et al. Antifungal susceptibilities, varieties, and electrophoretic karyotypes of clinical isolates of *Cryptococcus neoformans* from Brazil, Chile, and Venezuela. J Clin Microbiol 2001; 39: 2348–2350.
29. Christensen WB. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. J Bacteriol 1946; 52: 61–466.
30. Hopkins JM, Land GA. Rapid method for determining nitrate utilization by yeasts. J Clin Microbiol 1977; 5: 497–500.
31. Kwon-Chung KJ, Polacheck I, Bennett JE. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J Clin Microbiol 1982; 15: 535–537.
32. Weatherburn MW. Phenol-hypochlorite reaction for determination of ammonia. Anal Chem 1967; 39: 971–974.
33. Perfect JR, Magee BB, Magee PT. Separation of chromosomes of *Cryptococcus neoformans* by pulsed field gel electrophoresis. Infect Immun 1989; 57: 2624–2627.
34. Perfect JR, Netabchi N, Cox GM, Ingram CW, Beiser CL. Karyotyping of *Cryptococcus neoformans* as an epidemiological tool. J Clin Microbiol 1993; 31: 3305–3309.
35. Drouhet E. Milestones in the history of *Cryptococcus* and cryptococcosis. J Mycol Med 1997; 7: 10–27.
36. Irokanulo EO, Makinde AA, Akuesgi CO, Ekwonu M. *Cryptococcus neoformans* var. *neoformans* isolated from droppings of captive birds in Nigeria. J Wildl Dis 1997; 33: 343–345.
37. Bauwens L, Swinne D, De Vroey C, De Meurich W. Isolation of *Cryptococcus neoformans* var. *neoformans* in the aviaries of the Antwerp Zoological Garden. Mycosen 1986; 29: 291–294.
38. Vidotto V, Leone R, Sinicco A, Ito-Kuwa S, Criseo G. Comparison of phospholipase production in *Cryptococcus neoformans* isolates from AIDS patients and bird droppings. Mycopathologia 1998; 142: 71–76.
39. Vidotto V, Koga-Ito CY, Ito-Kuwa S et al. Extracellular activity in *Cryptococcus neoformans* strains isolated from AIDS patients and from environmental sources. Rev Iberoam Micol 2000; 17: 14–19.
40. Cox GM, McDade HC, Chen SCA, Tucker SC, Perfect JR et al. Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. Mol Microbiol 2001; 39: 166–175.
41. Cox GM, Mukherjee J, Cole GT, Casadevall A, Perfect J. Urease as a virulence factor in experimental cryptococcosis. Infect Immun 2000; 68: 443–448.

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ANEXO 5

HORTA, J. A.; FAGANELLO, J.; ROSA E SILVA, L. K.; OLIVEIRA, L. T.; SANTURIO, J. M.; VAINSTEIN, M. H. & ALVES, S. H. Susceptibility to heat and antifungal agents of *Cryptococcus neoformans* var. *neoformans* (serotype D) isolated from *Eucalyptus* spp. in Rio Grande do Sul, Brazil. *Braz J Microbiol*, 36:1-6, 2005.

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SUSCEPTIBILITY TO HEAT AND ANTIFUNGAL AGENTS OF *CRYPTOCOCCUS NEOFORMANS* VAR. *NEOFORMANS* (SEROTYPE D) ISOLATED FROM *EUCALYPTUS* SPP IN RIO GRANDE DO SUL, BRAZIL

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ABSTRACT

In this work we studied the susceptibility to heat and antifungal agents of the first strains of environmental *Cryptococcus neoformans* var *neoformans* (serotype D) isolated in the state of Rio Grande do Sul, Brazil. In order to achieve a rigorous analysis, we employed the methodology recommended by NCCLS, Yeast Nitrogen Base (YNB) proposed by Ghannoum *et al* (YNB-1), Antibiotic medium 3 (AM3) indicated by others, YNB adjusted to the NCCLS methodology (YNB-2) and Etest. Our results indicate that all strains were susceptible to amphotericin B (0.0625 – 0.5 µg/mL), fluconazole (0.125 – 8.0 µg/mL), itraconazole (0.031 – 0.25 µg/mL) and flucytosine (0.125 – 4.0 µg/mL). The *C. neoformans* serotype D strains were more susceptible to heat (47ºC / 30 min) than *C. neoformans* serotype A.

Key words: *Cryptococcus neoformans*, heat susceptibility, antifungal agents

INTRODUCTION

Cryptococcus neoformans, an encapsulated yeast, is the etiological agent of human cryptococcosis. In Brazil, 4.5% of all opportunistic infections in AIDS patients have been reported as being caused by *C. neoformans* (23). Based on physiological and serological differences, *C. neoformans* had been divided in two varieties: *C. neoformans* var. *neoformans* (serotypes A, D, AD) and *C. neoformans* var. *gattii* (serotypes B and C). On the basis of genetic differences it was proposed that *C. neoformans* var. *neoformans* be further subdivided in two varieties: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) (12).

The prevalence of the varieties and serotypes from either clinical or environmental sites differs in accordance with geographical localization. Environmental isolates of C. *neoformans* var. *grubii* from pigeons droppings indicate that serotype A is more common than serotype D in all nations, except Italy, Denmark and Switzerland (8) and have been recovered from approximately 99% of all patients in most countries (6,8,12). The serotype C is rarer than the other serotypes and has never been isolated from the environment. The majority of the few clinical isolates of serotype C have been found in southern California (8). The serotype B was initially isolated from *Eucalyptus camaldulensis* and *E. tereticornis* in Australia and more recently it has been isolated from other trees and immunocompromised (non- AIDS) patients in tropical and subtropical areas (8). In Rio Grande do Sul, *C. neoformans* var. *grubii* (serotype A) has been recovered from AIDS patients and pigeons excreta (1,6,14). The prevalence of *C. neoformans* var. *neoformans* (serotype D) among clinical isolates has ranged from 0 to 100% depending on the region of the world. In Brazil, the prevalence of serotype D is very low (29), and in the state of Rio Grande do Sul there were no

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records of clinical *C. neoformans* var. *neoformans* (serotype D) isolates.

Recently Ribeiro (28) isolated nine *C. neoformans* var. *neoformans* (serotype D) strains from 99 samples of *Eucalyptus* spp. As pointed out by Martinez *et al*. (21), the thermal sensibility of *C. neoformans* var. *neoformans* (serotype D) is a consistent explanation for the geographic differences between serotype A and D, and for the dermatotropism and rhinotropism observed. The purpose of this investigation was to assess the susceptibility of the first environmental *C. neoformans* var. *neoformans* (serotype D) isolates in Rio Grande do Sul, to heat and to a panel of antifungal agents commonly used in the treatment of infections. Because results of susceptibility tests based on M27-A2 methodology have been questioned due to factors such as suboptimal growth in RPMI 1640 medium and narrow amphotericin B MIC ranges (15,20), we decided to compare amphotericin B MICs obtained with RPMI 1640 medium to assays performed using Yeast Nitrogen Base proposed by Ghannoum *et al*. (YNB-1)(13), Antibiotic medium 3 (AM3) and Yeast Nitrogen Base supplemented medium adjusted to the M27-A2 methodology, which we named YNB-2 and the Etest method.

MATERIALS AND METHODS

Isolates

Porto Alegre, the capital city of the South Brazilian state Rio Grande do Sul is situated at 30° S latitude and 51° W longitude. The city is 10 m above the sea level with an average temperature of 22ºC and an annual rainfall of 1118 mm. Ninety-nine samples were collected from different *Eucalyptus* species (Table 1). Twenty other environmental strains of *C. neoformans* serotype A, isolated from pigeon excreta from Porto Alegre, Santa Cruz do Sul and Santa Maria (14), were also included in the analysis of heat susceptibility. All isolates were identified as *C. neoformans* by positive Niger seed agar response, as well as urease test, ability to grow at 35ºC and a negative nitrogen test. The profiles of carbon compound assimilation were also determined (4). Canavanine-glycine-bromothymol blue agar medium, assimilation of D-proline and D-tryptophan were used for differentiation of the two varieties (9) and serotyping was performed by a slide agglutination test (Crypto Check; Iatron Co; Japan).

Table 1. Origin of the environmental isolates of *Cryptococcus neoformans* (serotype D) isolated from *Eucalyptus* spp in Rio Grande do Sul, Brazil.

Location	Number of samples collected	Number of samples identified as *C. neoformans*
Barra do Ribeiro	53	0
Camaquã	8	0
Ijuí	3	0
Novo Hamburgo	2	2
Porto Alegre	7	5
São Leopoldo	2	2
São Lourenço do Sul	4	0
Sertão Santana	7	0
Soledade	3	0
Total	99	9

Susceptibility to heat (21)

A 1-ml aliquot of cells suspended at a density of 10^4 cells per ml in distilled water was incubated at 47ºC in water bath during 30 min. After incubation 20 µL of the cells suspension was plated onto Sabouraud dextrose agar and incubated for 48h at 30ºC. The survival percentage was determined by comparison to non-heat-treated control samples plated onto Sabouraud dextrose agar.

Antifungal susceptibility tests

The antifungal agents used were amphotericin B (Sigma), fluconazole (Pfizer), itraconazole (Jansen) and flucytosine (Sigma).

Test media were: RPMI 1640 (American Biorganics Inc.), indicated by the National Committee for Laboratory Standards (NCCLS) as reference, and Yeast Nitrogen base (Difco) were prepared according to manufacturer instructions. After reconstitution, both media were supplemented with glucose to obtain a final concentration of 0.5% in YNB (13) and 2% in RPMI 1640 (24). Both RPMI 1640 medium and YNB were buffered to pH 7.0 with 3-(N-morpholino) propanesulphonic acid (MOPS; Sigma, St. Louis, Mo, USA) to a final concentration of 165mM (NCCLS). Antibiotic medium 3 (AM3) (BBL) was supplemented with glucose to a final concentration of 2%; the buffering capacity was increased by adding 1g of dipotassium monophosphate per liter and 1g of monopotassium monophosphate per liter and pH was adjusted to 7.0 with NaOH (20). All three media were filter sterilized by using 0.22-µm membrane (Millipore).

The MICs of the tested agents were determined for each isolate in accordance with National Committee Laboratory Standards (NCCLS) macrodilution guidelines (24). The tubes were incubated at 35ºC and were read after 48h of incubation. The MIC of amphotericin B in RPMI 1640, YNB and AM3 was defined as the lowest concentration of drug that resulted in complete inhibition of visible growth. The tests were performed using two techniques with YNB: YNB-1 as indicated by Ghannoum *et al.* (13) and YNB-2 which use the same broth, but using the M27-A2 method (NCCLS). The MIC of azoles and flucytosine in RPMI 1640 were determined according to M27-A method (24). The data were reported as MIC ranges and MICs at which 50% and 90% of these isolates were inhibited. Quality

control testing was performed in accordance with NCCLS document M27-A2. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 (24) were used as quality control for the susceptibility tests.

Etest

To prepare the agar plates, the double-strength, filter-sterilized RPMI 1640 with 2% glucose was buffered with potassium phosphate at pH 7.0 and combined with an equal volume of heat-sterilized double-strength agar to yield the correct final concentration of medium in a 1.5% agar gel. The inoculum suspensions of *C. neoformans* strains matching the turbidity of McFarland #1 standard were swabbed onto the surface of the agar plate and allowed to dry for 15 min before the addition of the Etest strip (22). One Etest antimicrobial gradient strip was placed in each Petri dish. The plates were incubated for 48 and 72h, and the MIC was the point at which the zone of complete inhibition intersected the strip. Etest antimicrobial gradient (AB Biodisk, Solna, Sweden) strips containing amphotericin B, fluconazole, itraconazole and flucytosine were employed (32).

RESULTS

All isolates of *C. neoformans* var. *neoformans* (serotype D) grew well in RPMI 1640 broth, YNB-1, YNB-2, Antibiotic medium 3 and RPMI-1640 agar, allowing MICs to be determined after 72h incubation.

Table 2 summarizes the *in vitro* susceptibilities of the nine cultures tested by NCCLS method. The results are reported as MIC ranges, MIC_{50}s (50% of strains were inhibited) and MIC_{90}s (90% of strains were inhibited). A broad range of MICs was observed with fluconazole and flucytosine; more narrow MIC ranges were showed with amphotericin B and itraconazole. Table 3 summarizes the *in vitro* susceptibilities of the isolates to amphotericin B, as determined by NCCLS recommended medium, and other media suggested in the literature. All media employed showed similar MIC ranges, but YNB-1 showed slightly higher results. The MIC_{50} and MIC_{90} were the same for RPMI-1640, AM3 and YNB-2, but again higher for YNB-1. Based on the M27-A2 technique breakpoints, all the isolates were considered to be sensitive to the antifungal agents tested.

The MICs obtained by Etest are shown on Table 4. Itraconazole MICs are narrower in range, but amphotericin B, fluconazole and flucytosine had broad range of MICs. Based on $MIC_{50\%}$ or $MIC_{90\%}$, all the isolates were considered to be sensitive to the antifungal agents tested.

Among the 9 serotype D strains tested, 5 (55.5%) did not survive heat treatment and 4 (44.5%) showed percent survival varying between 22 and 76% when compared to the number of colonies in an equivalent suspension of cells not exposed to heat. When the 20 serotype A strains were assayed, only 3 (15%) did not grow on Sabouraud Dextrose agar after the thermal treatment; the range of percent survival was from 16 to 86%. The average percent survival of serotype D was 23.2%, while for serotype A it was 48%.

Table 2. Susceptibility of *Cryptococcus neoformans* serotype D to antifungal agents using the M27-A2 method.

Antifungal Agents	MIC[a] (µg/ml)			
	Range	50%	90%	GeoM[b]
Amphotericin B	0.0625-0.5	0.125	0.255	0.145
Fluconazole	0.125-8.0	1.0	4.0	1.46
Itraconazole	0.031-0.25	0.125	0.125	0.099
Flucytosine	0.125-4.0	0.5	2.0	0.793

[a]MICs for 50% and 90% of isolates tested; [b]geometric mean.
C. krusei ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control.

Table 3. Comparison of *in vitro* susceptibility of *Cryptococcus neoformans* serotype D strains to amphotericin B using in different media and assay types.

Assay types[a]	MIC[a] (µg/ml)			
	Range	50%[b]	90%[c]	GeoM[b]
RPMI	0.0625-0.5	0.125	0.25	0.134
AM3	0.0625-0.5	0.125	0.25	0.156
YNB-1	0.125-1.0	0.25	0.5	0.269
YNB-2	0.0625-0.5	0.125	0.25	0.170
E-test	0.0625-0.5	0.125	0.5	0.157

[a]See text for details; [b]50% and [c]90%, MICs for 50% and 90% of isolates tested, respectively; [d]GeoM; geometric mean.

Table 4. Susceptibility of *Cryptococcus neoformans* serotype D isolates to antifungal agents determined by using the Etest method.

Antifungal Agents	MIC[a] (µg/ml)			
	Range	50%	90%	GeoM[b]
Amphotericin B	0.0625-0.5	0.125	0.5	0.157
Fluconazole	0.25-8.0	2.0	4.0	1.851
Itraconazole	0.031-0.25	0.125	0.25	0.157
Flucytosine	0.125-4.0	1.0	2.0	0.857

[a]See text for details; [b]50% and [c]90%, MICs for 50% and 90% of isolates tested, respectively; [d]GeoM; geometric mean.

DISCUSSION

An understanding of the epidemiology of cryptococcosis may provide a rational framework for the design of prevention guidelines and more effective therapies. We believe that attempts to correlate specific environmental exposures to cryptococcal strains of known susceptibility profile may help to address some questions (14).

Based on these epidemiological statements, it is important to emphasize that cryptococcosis therapy may become even more difficult due the emergence of antifungal resistance. Amphotericin B (17,19), fluconazole (3,25,26,27), and flucytosine resistance have been well documented (30). Cross-resistance to both azole-types and amphotericin B has also been described (16).

In addition, recent articles reporting primary (intrinsic) resistance of *C. neoformans* var. *neoformans* (serotype D) to flucytosine (18) and, the isolation of fluconazole-resistant *C. neoformans* from an immunocompetent patient, without prior exposure to azoles (25), emphasizes the importance of carrying out susceptibility tests before beginning of therapy. This study reports the susceptibility testing of the first *C. neoformans* serotype D isolated from *Eucalyptus* spp. trees in Rio Grande do Sul state, employing additional methods for more accurate delineation of susceptibility profiles to antifungal agents.

The results of susceptibility tests to antifungal agents reported here are similar to those reported by others authors (7,15). However, some aspects deserve attention. In 2001, we have compared the susceptibility of clinical and environmental *C. neoformans* isolated in southern Brazil and observed that clinical isolates were less susceptible to fluconazole than environmental isolates, all of them of serotype A (1). Here, environmental strains showed a very similar pattern for amphotericin B and azoles, being all strains sensitive. One of the reasons to the absence of azole resistance might be the source of strains, which may have not had previous contact with azoles. The contrary has been reported in Italy, where clinical serotype D is prevalent. Tortorano *et al.* (31) showed that serotype A clinical strains were less susceptible to fluconazole than serotype D clinical isolates. This issue may deserve more attention.

The reference M27-A2 method based on RPMI 1640 medium has been used to test *C. neoformans* isolates (1,2,10,20). In spite of this indication, some previous reports suggested that RPMI 1640 medium did not support suitable growth of *C. neoformans*. In the present study, we have found that this medium, supplemented with 2% glucose, provided adequate growth of all strains tested; glucose supplementation is an alternative mentionated in the M27-A2 method (24). Due to reported concerns regarding amphotericin B resistance, in this study we have tested Antibiotic medium 3 (AM3), as indicated by Lozano-Chiu *et al.* (20), Yeast Nitrogen Base, as recommended by Ghannoum *et al.* (13), and also the same medium with changes in inoculum size and endpoint determination (YNB-2). The results presented have shown that the pattern of susceptibility to amphotericin B obtained with YNB-2, RPMI-1640 medium and AM3 was closely similar, with overlap of MIC ranges. YNB-1 resulted in a broader MIC range, though amphotericin B resistant strains were not detected. These results are similar to those already obtained from clinical strains of *C. neoformans* serotype A using the same media (2).

The MICs to flucytosine were low and so, based on established breakpoints, all strains were considered to be sensitive to this drug. This finding must be interpreted with caution, because approximately 2% of *C. neoformans* isolates are resistant to flucytosine prior to treatment (30). So, we must consider that the number of isolates studied was scarce, and DNA studies have shown that Brazilian *C. neoformans* isolates appeared to be less heterogenous than those isolated from other regions (11).

The Etest method performed on glucose-supplemented RPMI 1640 agar is an excellent method of discrimination between susceptible and resistant strains of *C. neoformans* (20,22,32). The susceptibility pattern obtained by Etest demonstrated a narrow range MICs to amphotericin B, and MIC_{50} and MIC_{90} one log dilution higher than the NCCLS method. For all antifungal agents studied, we have observed no significant changes on MICs by this method. This is in accordance with previous studies, that found complete or a high level of agreement between Etest and NCCLS method (5,22,32). The Etest has been considered an excellent method to distinguish amphotericin-B-resistant yeasts; it is reproducible, much simpler to set up than broth dilution methods and less labour-intensive. Thus, it has been recommended for routine use with amphotericin B and flucytosine (32).

Recently, Martinez *et al.* (21) analysed the heat susceptibility of 19 strains from each serotype group and observed a wide variation; *C. neoformans* serotype D strains being more susceptible. Our finds are in accordance with this study and corroborate what Dromer *et al.* (8) pointed out in that the differences in the prevalence of serotype A and D of *C. neoformans* may reflect climatic tolerances. The isolation of *C. neoformans* var. *neoformans* (serotype D) in Rio Grande do Sul (28), the southernmost and coolest state of Brazil, reflects the characteristics referred above, and might explain the rarity of this serotype in other areas of Brazil with sub-tropical and tropical climates. However, few studies were conducted and data are still scarce in this area (29).

RESUMO

Susceptibilidade de *Cryptococcus neoformans* var. *neoformans* (sorotipo D) isolados de *Eucalyptus* spp., no Rio Grande do Sul (Brasil), frente ao calor e a agentes antifúngicos

Este estudo foi realizado com os primeiros isolados ambientais de C. *neoformans* sorotipo D, obtidos no Rio Grande

do Sul. Objetivando-se avaliar a susceptibilidade a agentes antifúngicos de forma mais rigorosa, utilizou-se a técnica de referência proposta pelo NCCLS, Caldo Yeast Nitrogen Base (YNB) proposto por Ghannoum *et al.*, Antibiotic medium 3, caldo YNB adequado à metodologia do NCCLS e o E-test. Os resultados indicaram que todos os isolados foram sensíveis à anfotericina B (0,0625 -0,5 µg/mL), fluconazol (0,125 – 4,0 µg/mL), itraconazol (0,031 – 0,25 µg/ml) e fluorocitosina (0,125-4,0 µg/mL) através das técnicas empregadas. Nos testes de termotolerância (47ºC/30 min), observou-se que as culturas de *C. neoformans* sorotipo D são mais sensíveis do que as de C. *neoformans* sorotipo A.

Palavras-chave: *Cryptococcus neoformans,* susceptibilidade, antifúngicos

REFERENCES

1. Alves, S.H.; Oliveira, L.T.; Costa, J.M.; Lubeck, I.; Casali, A.K.; Vainstein, M.H. In vitro susceptibility to antifungal agents of clinical and environmental *Cryptococcus neoformans* isolated in southern of Brazil. *Rev. Inst. Med. Trop. São Paulo.*, 43(5), 267-270, 2001.
2. Alves, S.H.; Oliveira, L.T.; Goulart, L.S.; Linares, C.E.B.; Griebeler, J.; Santurio, J.M. Different culture media applied to the study of *Cryptococcus neoformans* susceptibility to amphotericin B and fluconazole. *Braz. J. Microbiol.*, 33, 27-30, 2002.
3. Armengou, A.; Pocar, C.; Mascaró, J.; Garcia-Bragado, F. Possible development of resistant to fluconazole during suppresive therapy for AIDS-associated cryptococcal meningitis. *Clin. Infect. Dis.*, 23(6), 1337-1338, 1996.
4. Barnett, J.A.; Payne, R.W.; Yarrow, D. *Yeasts: Characteristics and Identification,* 2nd Ed. Cambridge University Press, Cambridge, 1990. p.282-320.
5. Brandt, M.E.; Pfaller, M.A.; Hajjeh, R.A.; Hamill, R.J.; Pappas, P.G.; Reingold, A.L.; Rimland, D.; Warnock, D.W. Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolated in the United States: 1992 to 1994 and 1996 to 1998. *Antimicrob. Agents Chemother.*, 45(11), 3065-3069, 2001.
6. Casali, A.K.; Goulart, L.; Rosa e Silva, L.K.; Ribeiro, A.M.; Amaral, A.A.; Alves, S.H.; Schrank, A.; Meyer, W.; Vainstein, M.H. Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *FEMS Yeast Research.*, 3, 405-415, 2003.
7. Davey, K.G.; Holmes, A.D.; Johnson, E.M.; Szekely, A.; Warnock, D.W. Comparative evaluation of Fungitest and broth microdilution methods for antifungal susceptibility testing of *Candida* species and *Cryptococcus neoformans*. *J. Clin. Microbiol.*, 36(4): 926-930, 1998.
8. Dromer, F.; Mathaulin, S.; Dupont, B.; Letenneur, L.; Ronin, O.; The French Cryptococcosis Study Group. Individual and environmental factors associated with infection due to *Cryptococcus neoformans* serotype D. *Clin. Infec. Dis.*, 23, 91-96, 1996.
9. Dufait, R.; Velho, R.; DeVroey, C. Rapid identification of the two varieties of *Cryptococcus neoformans* by D-proline assimilation. *Mykosen.*, 30, 483, 1987.
10. Franzot, S.P.; Hamdan, J.S. *In vitro* susceptibilities of clinical and environmental isolates of *Cryptococcus neoformans* to five antifungal agents. *Antimicrob. Agents Chemother.*, 40, 822-824, 1996.
11. Franzot, S.P.; Hamdan, J.A.; Currie, B.P.; Casadevall, A. Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: evidence for both local genetic differences and a global population structure. *J. Clin. Microbiol.*, 35(9), 2243-2251, 1997.
12. Franzot, S.P.; Salkin, I.F.; Casadevall, A. *Cryptococcus neoformans* var *grubii:* separate varietal status of *C. neoformans* serotypes A Isolates. *J. Clin. Microbiol.*, 37, 838-840, 1999.
13. Ghannoum, M.A.; Ibrahim, A.S.; Fu, Y.; Schafiq, M.; Edwards, J.E.; Criddle, R.S. Susceptibility testing of *Cryptococcus neoformans*: a microdilution technique. *J. Clin. Microbiol.*, 30, 2881-2886, 1992.
14. Horta, J.A.; Staats, C.C.; Casali, A.K.; Ribeiro, A.M.; Schrank, I.S.; Schrank, A.; Vainstein, M.H. Epidemiological aspects of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state of Rio Grande do Sul. *Med. Mycol.*, 40, 565-571, 2002.
15. Jessup, C.J.; Pfaller, M.A.; Messer, S.A.; Zhang, J.; Tumberland, M.; Mbidde, E.K.; Ghannoum, M.A. Fluconazole susceptibility testing of *Cryptococcus neoformans*: comparison of two broth microdilution methods and clinical correlates among isolates fron Ugandan AIDS patients. *J. Clin. Microbiol.*, 36(10), 2874-2876, 1998.
16. Joseph-Horne, T.; Hollomon, D.; Loeffler, R.S.T.; Kelly, S.L. Cross-resistance to polyene and azole drug in *Cryptococcus neoformans*. *Antimicrob Agents Chemother.*, 39, 1526-1529, 1995.
17. Joseph-Horne, T.; Loeffler, R.S.T.; Hollomon, D.W.; Kelly, S.L. Amphotericin B resistant isolates of *Cryptococcus neoformans* without alteration in sterol biosynthesis. *J. Med. Vet. Mycol.*, 34, 223-225, 1996.
18. Kantarcioglu, A.S.; Yucel, A. A flucytosine-resistant *Cryptococcus neoformans* (serotype D) strains isolated in Turkey from cutaneous lesions. *Med. Mycol.*, 40, 519-523, 2002.
19. Kelly, S.L.; Lamb, D.C.; Taylor, M.; Corran, A.J.; Baldwin, B.C.; Powderly, W.G. Resistance to amphotericin B associated with defective sterol Δ 8.7 isomerase in a *Cryptococcus neoformans* strains from AIDS patients. *FEMS Microbiol. Lett.*, 122, 39-42, 1994.
20. Lozano-Chiu, M.; Paetznick, V.L.; Ghannoum, M.A.; Rex, J.H. Detection of resistance to amphotericin B among *Cryptococcus neoformans* clinical isolates: performances of three different media assessed by using E-test and National Committee for Clinical laboratory Standards M27-A methodologies. *J. Clin. Microbiol.*, 36 (10), 2817-2822, 1998.
21. Martinez, L.R.; Garcia-Rivera, J.; Casadevall, A. *Cryptococcus neoformans* var *neoformans* (serotype D) strains are more susceptible to heat than *C. neoformans* var *grubii* (serotype A) strains. *J. Clin. Microbiol.*, 39(9), 3365-3367, 2001.
22. Maxwell, M.J.; Messer, S.A.; Hollis, R.J.; Diekema, D.J.; Pfaller, M.A. Evaluation of Etest method for determining voriconazole and amphotericin B MICs for 162 clinical isolates of *Cryptococcus neoformans*. *J. Clin. Microbiol.*, 41(1), 97-99, 2003.
23. Ministério da Saúde. Programa nacional de doenças sexualmente transmissíveis, Brasília. *Brasil Bol. Epidemiol. AIDS*, 1(1), 44, 1999.
24. *NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS*. Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard M27-A2. National Committee for Clinical laboratory Standards, Wayne, Pa. 2002.
25. Orni-Wasserlauf, R.; Izhakov, E.; Siegman-Igray, Y.; Bash, E.; Polacheck, I.; Giladi, M. Fluconazole-resistant *Cryptococcus neoformans* isolated from an immunocompromised patient without prior exposure to fluconazole. *Clin. Infect. Dis.*, 29, 11592-1593, 1999.
26. Paugham, A.; Dupoy-Camet, J.; Blanche, P.; Gangneux, J.P.; Tourte-Schaefer, C.; Sicard, D. Increased fluconazole resistance of *Cryptococcus neoformans* isolated from a patient with AIDS and recurrent meningitis. *Clin. Infect. Dis.*, 19, 976-977, 1994.
27. Peetermans, W.; Bobbaers, H.; Verhaegen, J.; Vandepitte, J. Fluconazole-resistant *Cryptococcus neoformans* var *gattii* in an AIDS patient. *Acta Clin. Belg.*, 48, 405-409, 1993.

28. Ribeiro, A.M. *Isolamento e Caracterização de Cryptococcus neoformans* a partir de *Eucalyptus spp. no Rio Grande do Sul.* [Dissertação de Mestrado]. Programa de Pós-graduação em Biologia Celular e Molecular; Centro de Biotecnologia do Estado do Rio Grande do Sul. Universidade Federal do Rio Grande do Sul, 2002. p.67.
29. Rozembaum, R.A.; Gonçalves, A.J.R.; Wanke, B.; Caiuby, M.J.; Clemente, H.; Lazera, M.S.; Monteiro, P.C.F.; Londero, A.T. *Cryptococcus neoformans* varieties as agents of cryptococcosis in Brazil. *Mycopathologia*, 199, 133-136, 1992.
30. Scholer, H.J.; Polak, A. Resistance to systemic antifungal agents. *In:* Bryan, L.E.; ed. *Antimicrobial Drug Resistance*. Orlando, Academic Press, 1984, p.393-460.
31. Tortorano, A.M.; Viviani, M.A.; Rigoni, A.L.; Cogliati, M.; Roverseli, A.; Pagano, A. Prevalence of serotype D in *Cryptococcus neoformans* isolates from HIV positive and HIV negative patients in Italy. *Mycoses.*, 40, 297-302, 1997.
32. Warnock, D.W.; Johnson, E.M.; Rogers, T.R.F.; and behalf of the BSAC Working Party on Antifungal Chemotherapy. *J. Antimicrob. Chemother.*, 42, 321-331, 1998.