Simultaneous identification of molecular and mating types within the *Cryptococcus* species complex by PCR-RFLP analysis

Xiaobo Feng,¹ Zhirong Yao,¹ Daming Ren² and Wanqing Liao³

Correspondence

Zhirong Yao

zryaosmu@sohu.com

¹Medical Mycology Laboratory, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, PR China

²State Key Laboratory of Genetic Engineering, Institute of Genetics, Fudan University, Shanghai, PR China

³Medical Mycology Laboratory, Shanghai Changzheng Hospital, Shanghai, PR China

The *Cryptococcus* species complex consists of two species, *Cryptococcus neoformans* and *Cryptococcus gattii*, which cause systemic infections in both immunocompromised and immunocompetent patients. Both species have a bipolar mating system, with mating type (*MAT*) α being predominant in clinical and environmental isolates. The strains of the *Cryptococcus* species complex have been divided into eight major molecular types, which show differences in epidemiology, biology and pathogenicity. In this study, two PCR-RFLP analyses, based on the *CAP1* and *GEF1* genes, which are both located at the *MAT* locus, were developed for simultaneous identification of the molecular and mating types of isolates of the *Cryptococcus* species complex. The molecular and mating types of all 144 cryptococcal isolates, including rare subtypes, were successfully determined by both PCR-RFLP approaches. Pattern analysis of the AD hybrids revealed that the serotype A *MAT* α allele in strains of AaD α derived from genotype VNB, whereas the serotype A *MAT* α allele among strains of AxDa and AxD α derived from molecular type VNI.

Received 21 May 2008

Accepted 29 July 2008

INTRODUCTION

The *Cryptococcus* species complex contains the most common causative agents of fungal meningoencephalitis in immunocompromised patients, particularly in the AIDS population (Mitchell & Perfect, 1995; Casadevall & Perfect, 1998). Within the *Cryptococcus* species complex, two species, *Cryptococcus neoformans* and *Cryptococcus gattii*, have been named. Both species have a bipolar mating system involving α and *a* mating types (*MAT*) (Kwon-Chung *et al.*, 2002; Kwon-Chung & Varma, 2006). The *Cryptococcus* species complex is currently divided into eight major molecular types: VNI/AFLP1 and VNII/AFLP1A/1B (*C. neoformans* var. *grubii*, serotype A); VNIII/AFLP3 (AD hybrid, serotype AD); VNIV/AFLP2 (*C. neoformans* var. *neoformans*, serotype D); and VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5 and VGIV/AFLP7 (*C. gattii*, serotypes B and C) (Boekhout *et al.*, 2001; Latouche *et al.*, 2003; Meyer *et al.*, 2003; Bovers *et al.*, 2008a). In addition to the eight established molecular types with opposite mating types, a genotype, VNB, unique to Botswana and a few novel

hybrids of AxD α and *C. neoformans* $\times$ *C. gattii* have been described in recent studies (Litvintseva *et al.*, 2005a, 2006; Bovers *et al.*, 2006; Lin *et al.*, 2007; Bovers *et al.*, 2008b). Differences in biology, epidemiology, pathogenicity, clinical features and drug susceptibility have been reported to be associated with species, varieties, molecular types and mating types within the *Cryptococcus* species complex (Kwon-Chung *et al.*, 1992; Speed & Dunt, 1995; Casadevall & Perfect, 1998; Meyer *et al.*, 2003; Trilles *et al.*, 2004; Campbell *et al.*, 2005; Fraser *et al.*, 2005).

C. neoformans is a ubiquitous pathogen associated with avian faeces and decaying wood, being responsible for most cases of cryptococcosis. The most common variety, *C. neoformans* var. *grubii*, primarily infects immunocompromised individuals and accounts for most cryptococcal infections (>90 %) (Casadevall & Perfect, 1998). Strains of serotype A *MAT* α (Ax) have been shown to be more pathogenic than Aa strains in several reports (Lengeler *et al.*, 2000; Keller *et al.*, 2003; Barchiesi *et al.*, 2005). Despite molecular types VNI and VNII both appearing to be distributed globally, VNII is recovered less frequently (Meyer *et al.*, 2003). In addition, a unique genotype, VNB, belonging to *C. neoformans* var. *grubii* has recently been found to be restricted to Botswana. VNB includes a

The GenBank/EMBL/DDBJ accession numbers for the *CAP1* and *GEF1* sequences for the reference strains of WM626, CBS1930, WM161, B4546 and WM779 are EU571727–EU571731 and EU571732–EU571736, respectively.

large proportion of fertile isolates of *MATa* (Litvintseva *et al.*, 2006). The second variety, *C. neoformans* var. *neoformans*, causing cases mainly in Europe, has been reported to be less virulent and more susceptible to fluconazole than *C. neoformans* var. *grubii* (Tortorano *et al.*, 1997; Casadevall & Perfect, 1998). In murine models, *D α* strains are more virulent than congenic *Da* strains (Kwon-Chung *et al.*, 1992). AD strains are hybrids between the two varieties, and possess two mating type alleles with one from each of the varieties. It is suggested that AD hybrids may be more common in clinical and environmental sources than was previously thought (Litvintseva *et al.*, 2005a; Viviani *et al.*, 2006). Further studies have shown that AD hybrids exhibit hybrid vigour in the laboratory (Lin *et al.*, 2007; Litvintseva *et al.*, 2007). Interestingly, strains of *AzDa* have been reported to be more virulent than *AaD α* strains (Barchiesi *et al.*, 2005). Moreover, a hybrid genotype of *AzD α* has recently been recovered from the USA and Italy, implicating same-sex mating between α mating types within this species (Litvintseva *et al.*, 2005a; Lin *et al.*, 2007).

C. gattii, previously known as *C. neoformans* var. *gattii*, has been raised to species level (Kwon-Chung *et al.*, 2002). This sibling species was primarily restricted to tropical and subtropical climates until an outbreak on Vancouver Island, Canada, in 2000. It inhabits decaying wood of eucalypts or other tree species and primarily infects immunocompetent individuals (Casadevall & Perfect, 1998; Kidd *et al.*, 2004). Compared with *C. neoformans*, the clinical characteristics of *C. gattii* have been reported to be different. It is the cause of cryptococcomas and has a lower susceptibility to several antifungal agents, a need for prolonged treatment and a higher mortality rate (Speed & Dunt, 1995; Trilles *et al.*, 2004). Like *C. neoformans*, isolates of *C. gattii* with the *MAT α* mating type are also predominant in clinical and environmental samples, especially in the former (Campbell *et al.*, 2005; Fraser *et al.*, 2005; Litvintseva *et al.*, 2005b). Among the four previously defined molecular types of *C. gattii*, molecular types VGI and VGII are widely distributed, whereas VGIII and VGIV are usually isolated from tropical climates (Meyer *et al.*, 2003; Litvintseva *et al.*, 2005b). Strains of VGII *MAT α* have been revealed to be related to a high incidence of *C. gattii* infections, such as the cryptococcosis outbreak on Vancouver Island (Kidd *et al.*, 2004; Campbell *et al.*, 2005; Fraser *et al.*, 2005). Strains of VGIV *MAT α* have been recovered mainly from patients with AIDS in sub-Saharan Africa (Litvintseva *et al.*, 2005b). Furthermore, several novel *C. gattii* $\times$ *C. neoformans* hybrids in which the *C. gattii* alleles originated from the molecular type VGI have been described in clinical samples (Bovers *et al.*, 2006, 2008b).

The population structure of the eight molecular types has been confirmed by sequence and phylogenetic analyses of multiple genes (Fraser *et al.*, 2005; Bovers *et al.*, 2008a). RFLP analysis based on a single gene was developed for the differentiation of the molecular types and confirmed to be a reliable and easy-to-use method (Latouche *et al.*, 2003;

Meyer *et al.*, 2003). In a recent study, sequence analysis of the *CAP1* gene, which is located at the *MAT* locus, indicated that sequence variation due to the mating type was greater than that due to the molecular type divergence. The dendrogram showed that strains clustered according to mating type, and in each of the two clades, strains clustered according to molecular type (Kidd *et al.*, 2005). In a previous study by us, partial sequences of the *GEF1* gene (a gene also located at the *MAT* locus) for strains representative of the distinct molecular and mating types were determined. The results showed that this gene exhibited genetic traits similar to those of the *CAP1* gene (X. Feng and others, unpublished results). These data demonstrated that the molecular and mating types of strains belonging to the *Cryptococcus* species complex could be determined simultaneously by sequencing either of the two genes.

In the present study, two alternative approaches using PCR-RFLP analysis, based on the *CAP1* and *GEF1* genes, requiring only common materials in the laboratory were developed to determine simultaneously the molecular and mating types of isolates of the *Cryptococcus* species complex.

METHODS

Strains and DNA extraction. One hundred and forty-four isolates of the *Cryptococcus* species complex comprising 96 *C. neoformans* isolates and 48 *C. gattii* isolates were used in this study. Among the tested strains, 135 represented the 14 distinct groups of the molecular and mating types, namely VNI *MAT α* , VNI *MATa*, VNII *MAT α* , VNII *MATa*, VNIII *MAT α* , VNIII *MATa*, VNIV *MAT α* , VNIV *MATa*, VGI *MAT α* , VGI *MATa*, VGII *MAT α* , VGII *MATa*, VGIII *MAT α* , VGIII *MATa* and VGIV *MAT α* . The other nine isolates represented the rare subtypes of the genotype VNB (including *MAT α* and *MATa*) and the AD hybrid of *AzD α* . The data for the strains tested are shown in Table 1. The molecular and mating types of 69 isolates were determined previously in other studies, and those of the other 75 isolates (Table 1) were determined by PCR fingerprinting (Meyer *et al.*, 2003) and PCR analysis with primers specific for the *STE20* and *STE12* genes situated within the *MAT* locus as described previously by Lengeler *et al.* (2001) and Fraser *et al.* (2003), respectively. The strains recovered from China were identified as *Cryptococcus* species by morphology and biochemical and physiological tests, such as microscopic observation of India ink preparation, black or brown colony appearance on L-3,4-dihydroxy-phenylalanine agar, a positive urease reaction and the ability to grow at 37 °C (X. Feng and others, unpublished results). Strains were maintained on yeast extract-peptone-glucose medium at 30 °C for 2 days before testing. Genomic DNA was prepared from each strain as described previously (Meyer *et al.*, 2003).

PCR amplification. The sequences of the *CAP1* and *GEF1* genes from the reference strains representing distinct molecular and mating types, including H99 (VNI *MAT α* ; GenBank accession no. AF542529), 125.91 (VNI *MATa*; AF542528), JEC21 (VNII *MAT α* ; AF542531), JEC20 (VNII *MATa*; AF542530), WM276 (VGI *MAT α* ; AY710430), E566 (VGI *MATa*; AY710429) and R265 (VGII *MAT α* ; DQ096809), were obtained from GenBank. The sequences for the reference strains of WM626 (VNII *MAT α*), CBS1930 (VGII *MATa*), WM161 (VGIII *MAT α*), B4546 (VGIII *MATa*) and WM779 (VGIV *MAT α*) were generated in our study using TA cloning and a nested sequencing approach. Primers were designed from the consensus sequence based on alignment of the targeted gene sequences from the above reference strains. For the *CAP1* gene, two primers (*CAP1F*, 5'-

Table 1. Strains of the *Cryptococcus* species complex tested in this study and RFLP results obtained for the *CAP1* and *GEF1* genes

Strain	Origin*	Serotype	Molecular type	Mating type	RFLP pattern		Reference/source
					CAP1	GEF1	
<i>C. neoformans</i>							
<i>var. grubii</i>							
H99	CSF, USA	A	VNI	α	C1	G1	Lengeler <i>et al.</i> (2001); J. R. Perfect
WM148	CSF, Australia	A	VNI	α	C1	G1	Kidd <i>et al.</i> (2004); W. Meyer
MAS92-0006	USA	A	VNI	α	C1	G1	J. P. Xu
RV45886	Belgium	A	VNI	α	C1	G1	‡; C. De Vroey
RV67754	Belgium	A	VNI	α	C1	G1	This study; C. De Vroey
RV67746	Belgium	A	VNI	α	C1	G1	This study; C. De Vroey
XH95	Clinical, China	A	VNI	α	C1	G1	†
XH109	Clinical, China	A	VNI	α	C1	G1	†
XH89	Clinical, China	A	VNI	α	C1	G1	†
XH67	Clinical, China	A	VNI	α	C1	G1	†
XH1	Clinical, China	A	VNI	α	C1	G1	†
XH108	Clinical, China	A	VNI	α	C1	G1	†
XH37	Clinical, China	A	VNI	α	C1	G1	†
XH102	Clinical, China	A	VNI	α	C1	G1	†
XH81	Clinical, China	A	VNI	α	C1	G1	†
XH116	Clinical, China	A	VNI	α	C1	G1	†
XH99	Clinical, China	A	VNI	α	C1	G1	†
XH83	Clinical, China	A	VNI	α	C1	G1	†
XH103	Clinical, China	A	VNI	α	C1	G1	†
XH113	Clinical, China	A	VNI	α	C1	G1	†
XH114	Clinical, China	A	VNI	α	C1	G1	†
XH22	Clinical, China	A	VNI	α	C1	G1	†
XH110	Clinical, China	A	VNI	α	C1	G1	†
XH94	Clinical, China	A	VNI	α	C1	G1	†
XH84	Clinical, China	A	VNI	α	C1	G1	†
XH105	Clinical, China	A	VNI	α	C1	G1	†
XH40	Clinical, China	A	VNI	α	C1	G1	†
XH80	Clinical, China	A	VNI	α	C1	G1	†
XH33	Clinical, China	A	VNI	α	C1	G1	†
XH41	Clinical, China	A	VNI	α	C1	G1	†
XH93	Clinical, China	A	VNI	α	C1	G1	†
XH39	Clinical, China	A	VNI	α	C1	G1	†
XH55	Clinical, China	A	VNI	α	C1	G1	†
XH106	Clinical, China	A	VNI	α	C1	G1	†
XH107	Clinical, China	A	VNI	α	C1	G1	†
XH104	Clinical, China	A	VNI	α	C1	G1	†
XH21	Clinical, China	A	VNI	α	C1	G1	†
XH28	Clinical, China	A	VNI	α	C1	G1	†
XH7	Clinical, China	A	VNI	α	C1	G1	†
XH70	Clinical, China	A	VNI	α	C1	G1	†
XH68	Clinical, China	A	VNI	α	C1	G1	†
XH85	Clinical, China	A	VNI	α	C1	G1	†
XH82	Clinical, China	A	VNI	α	C1	G1	†
XH65	Clinical, China	A	VNI	α	C1	G1	†
XH76	Clinical, China	A	VNI	α	C1	G1	†
XH92	Clinical, China	A	VNI	α	C1	G1	†
XH59	Clinical, China	A	VNI	α	C1	G1	†
XH43	Clinical, China	A	VNI	α	C1	G1	†
XH42	Clinical, China	A	VNI	α	C1	G1	†
XH79	Clinical, China	A	VNI	α	C1	G1	†
XH87	Clinical, China	A	VNI	α	C1	G1	†

Table 1. cont.

Strain	Origin*	Serotype	Molecular type	Mating type	RFLP pattern		Reference/source
					CAPI	GEFI	
IUM96-2828	Environmental, Italy	A	VNI	a	C2	G2	Litvintseva <i>et al.</i> (2007)
KN99a	Laboratory, USA	A	VNI	a	C2	G2	Lin <i>et al.</i> (2007)
bt130	Clinical, Botswana	A	VNI	a	C2	G2	Litvintseva <i>et al.</i> (2006)
WM626	CSF, Australia	A	VNII‡	α	C3	G3	Kidd <i>et al.</i> (2004); W. Meyer
c12	Lung, USA	A	VNII	α	C3	G3	Litvintseva <i>et al.</i> (2006)
c45	Sputum, USA	A	VNII	α	C3	G3	Litvintseva <i>et al.</i> (2006)
A7 35-23	Pigeon excreta, USA	A	VNII	α	C3	G3	Litvintseva <i>et al.</i> (2006)
571-152	Cat, Australia	A	VNII	α	C3	G3	Bui <i>et al.</i> (2008)
571-154	Cat, Australia	A	VNII	α	C3	G3	Bui <i>et al.</i> (2008)
571-160	Cat, Australia	A	VNII	α	C3	G3	Bui <i>et al.</i> (2008)
bt34	CSF, Botswana	A	VNB	α	C1	G15	Litvintseva <i>et al.</i> (2006)
bt84	CSF, Botswana	A	VNB	α	C1	G15	Litvintseva <i>et al.</i> (2006)
bt60	CSF, Botswana	A	VNB	α	C1	G15	Litvintseva <i>et al.</i> (2006)
bt88	CSF, Botswana	A	VNB	a	C15	G2	Litvintseva <i>et al.</i> (2006)
bt206	CSF, Botswana	A	VNB	a	C15	G2	Litvintseva <i>et al.</i> (2006)
AD hybrids							
RV53164	Belgium	AD	VNIII	α/a	C4	G4	Cogliati <i>et al.</i> (2001)
YD38	Italy	AD	VNIII	α/a	C4	G4	This study; M. A. Viviani
WM628	CSF, Australia	AD	VNIII	α/a	C4	G4	Kidd <i>et al.</i> (2004); W. Meyer
MMRL774	Clinical, USA	AD	VNIII	α/a	C4	G4	Lengeler <i>et al.</i> (2001)
ICB134	Pigeon, Brazil	AD	VNIII	α/a	C4	G4	C. R. Paula
RV33370	Clinical, Belgium	AD	VNIII	α/a	C4	G4	Cogliati <i>et al.</i> (2001)
XH118	CSF, China	AD	VNIII	α/a	C4	G4	This study
CDC228	USA	AD	VNIII	a/α	C5	G5	Lengeler <i>et al.</i> (2001)
ATCC48184	Avian, Japan	AD	VNIII	a/α	C5	G5	Lengeler <i>et al.</i> (2001)
IUM92-6198	Italy	AD	VNIII	a/α	C5	G5	Lin <i>et al.</i> (2007)
ZG290	Clinical, China	AD	VNIII	a/α	C5	G5	Lengeler <i>et al.</i> (2001)
MMRL1365	Clinical, USA	AD	VNIII	a/α	C5	G5	Litvintseva <i>et al.</i> (2007)
5-19	Environmental, USA	AD	VNIII	α/α	C16	G16	Lin <i>et al.</i> (2007)
6-20	Environmental, USA	AD	VNIII	α/α	C16	G16	Lin <i>et al.</i> (2007)
713	Italy	AD	VNIII	α/α	C16	G16	Lin <i>et al.</i> (2007)
42-10	Environmental, USA	AD	VNIII	α/α	C16	G16	Lin <i>et al.</i> (2007)
var. neoformans							
RV51276	Clinical, France	D	VNIV	α	C6	G6	This study; C. De Vroey
JEC21	Laboratory, USA	D	VNIV	α	C6	G6	Lengeler <i>et al.</i> (2001)
RV60047	Skin, Belgium	D	VNIV	α	C6	G6	This study; C. De Vroey
WM629	Blood, Australia	D	VNIV	α	C6	G6	Kidd <i>et al.</i> (2004); W. Meyer
YD26	Italy	D	VNIV	α	C6	G6	This study; M. A. Viviani
CDC92-18	USA	D	VNIV	α	C6	G6	Lengeler <i>et al.</i> (2002)
RV45980	Laboratory, USA	D	VNIV	α	C6	G6	C. De Vroey
JEC20	Laboratory, USA	D	VNIV	a	C7	G7	Lengeler <i>et al.</i> (2001)
NIH430	Clinical, Denmark	D	VNIV	a	C7	G7	K. J. Kwon-Chung
NIH433	Environmental, Denmark	D	VNIV	a	C7	G7	K. J. Kwon-Chung
B-3502	Laboratory, USA	D	VNIV	a	C7	G7	K. J. Kwon-Chung
YD53	Italy	D	VNIV	a	C7	G7	This study; M. A. Viviani
XH56	Clinical, China	D	VNIV	a	C7	G7	†
XH117	CSF, China	D	VNIV	a	C7	G7	This study
C. gattii							
WM179	CSF, Australia	B	VGI	α	C8	G8	Kidd <i>et al.</i> (2004); W. Meyer
WM276	<i>Eucalyptus</i> , Australia	B	VGI	α	C8	G8	Campbell <i>et al.</i> (2005); W. Meyer
B5242	Clinical, USA	B	VGI	α	C8	G8	Fraser <i>et al.</i> (2005)
E286	Environmental, Australia	B	VGI	α	C8	G8	Fraser <i>et al.</i> (2005); W. Meyer
RV66095	CSF, Brazil	B	VGI	α	C8	G8	‡; C. De Vroey
RV3463	CSF, Belgium	B	VGI	α	C8	G8	‡; C. De Vroey

Table 1. cont.

Strain	Origin*	Serotype	Molecular type	Mating type	RFLP pattern		Reference/source
					CAP1	GEF1	
RV66536	Clinical, Brazil	B	VGI	α	C8	G8	†; C. De Vroey
XH111	Mouse brain, China	B/C	VGI	α	C8	G8	This study
RV8001	CSF, Belgium	B	VGI	α	C8	G8	†; C. De Vroey
RV52641	Belgium	B/C	VGI	α	C8	G8	†; C. De Vroey
XH3	Clinical, China	B/C	VGI	α	C8	G8	†
XH4	Clinical, China	B/C	VGI	α	C8	G8	†
XH5	Clinical, China	B/C	VGI	α	C8	G8	†
XH23	Clinical, China	B/C	VGI	α	C8	G8	†
XH24	Clinical, China	B/C	VGI	α	C8	G8	†
XH31	Clinical, China	B/C	VGI	α	C8	G8	†
XH32	Clinical, China	B/C	VGI	α	C8	G8	†
XH72	Clinical, China	B/C	VGI	α	C8	G8	†
E566	<i>Eucalyptus</i> , Australia	B	VGI	a	C9	G9	Campbell <i>et al.</i> (2005); W. Meyer
E276	<i>Eucalyptus</i> , Australia	B	VGI	a	C9	G9	Fraser <i>et al.</i> (2005); W. Meyer
E312	<i>Eucalyptus</i> , Australia	B	VGI	a	C9	G9	Fraser <i>et al.</i> (2005); W. Meyer
E554	<i>Eucalyptus</i> , Australia	B	VGI	a	C9	G9	Fraser <i>et al.</i> (2005); W. Meyer
C2077	Environmental, Australia	B/C	VGI	a	C9	G9	Fraser <i>et al.</i> (2005)
NT-2	Clinical, Australia	B	VGI	a	C9	G9	Fraser <i>et al.</i> (2005)
NIH444	Clinical, USA	B	VGII	α	C10	G10	Fraser <i>et al.</i> (2005)
R265	Clinical, Vancouver	B	VGII	α	C10	G10	Fraser <i>et al.</i> (2005); J. Kronstad
ICB179	Environmental, Brazil	B	VGII	α	C10	G10	Fraser <i>et al.</i> (2005)
WM178	Lung, Australia	B	VGII	α	C10	G10	Kidd <i>et al.</i> (2004); W. Meyer
XH91	Clinical, China	B/C	VGII	α	C10	G10	†
R272	Clinical, Vancouver	B	VGII	α	C10	G10	Fraser <i>et al.</i> (2005); J. Kronstad
2003/125	Clinical, France	B	VGII	α	C10	G10	Fraser <i>et al.</i> (2005)
CBS1930	Veterinary, Aruba	B	VGII	a	C11	G11	Fraser <i>et al.</i> (2005); T. Boekhout
WM161	<i>Eucalyptus</i> , USA	B	VGIII	α	C12	G12	Kidd <i>et al.</i> (2004); W. Meyer
NIH179	Clinical	B	VGIII	α	C12	G12	Fraser <i>et al.</i> (2005)
MAS92-0409	USA	B	VGIII	α	C12	G12	J. P. Xu
NIH836	Clinical	C	VGIII	α	C12	G12	Fraser <i>et al.</i> (2005)
NIH312	CSF, USA	C	VGIII	α	C12	G12	Campbell <i>et al.</i> (2005)
RV45184	Belgium	B/C	VGIII	α	C12	G12	†; C. De Vroey
B4546	Clinical	C	VGIII	a	C13	G13	Campbell <i>et al.</i> (2005); W. Schell
NIH191	Clinical, USA	C	VGIII	a	C13	G13	Fraser <i>et al.</i> (2005)
NIH198	Clinical	B	VGIII	a	C13	G13	K. J. Kwon-Chung
ICB108	Clinical, Brazil	C	VGIII	a	C13	G13	Fraser <i>et al.</i> (2005)
WM779	Cheetah, South Africa	C	VGIV‡	α	C14	G14	Kidd <i>et al.</i> (2004); W. Meyer
MMRL2872	Clinical, Botswana	C	VGIV	α	C14	G14	Litvintseva <i>et al.</i> (2005b)
MMRL2874	Clinical, Botswana	C	VGIV	α	C14	G14	Litvintseva <i>et al.</i> (2005b)
MMRL2980	Clinical, Botswana	C	VGIV	α	C14	G14	Litvintseva <i>et al.</i> (2005b)
MMRL2933	Clinical, Botswana	C	VGIV	α	C14	G14	Litvintseva <i>et al.</i> (2005b)
YD48	Italy	B/C	VGIV	α	C14	G14	†; M. A. Viviani

*CSF, cerebrospinal fluid.

†The molecular types and/or mating types were determined in a previous study by us by PCR fingerprinting and/or PCR analysis of the *STE20* and *STE12* genes, respectively (X. Feng and others, unpublished results).‡No mating type **a** within these molecular types has been reported until now.

TCAGATCAACCGCGACATCG-3'; and CAP1R, 5'-TGAACCC-AAGGGGTAAGCCA-3') comprising nt 664–683 and nt 1879–1898, respectively, based on the *CAP1* sequence of strain JEC21 were designed to amplify a portion of the *CAP1* gene from all strains investigated (Fig. 1a). For the *GEF1* gene, two primers (GEF1F, 5'-

GGACCCATGCCTGAAATGTG-3'; and GEF1R, 5'-TACGCTT-GCCCCGATCTG-3') comprising nt 1703–1722 and nt 3024–3041, respectively, based on the *GEF1* sequence of the strain JEC21 were designed to amplify a portion of the *GEF1* gene from all strains tested (Fig. 1b).

Fig. 1. Schematic representation of the amplified regions of the *CAP1* gene (a) and *GEF1* gene (b). Primer binding sites are shown by short arrows. Open boxes represent exons, with the order indicated by numbers, whilst lines between exons represent introns.

Amplification was performed in 1 × PCR buffer containing 1.5 mM MgCl₂, 0.2 mM each dNTP, 10 μM each primer and 1.5 U *Taq* polymerase. The PCR conditions for both amplifications were: initial denaturation at 94 °C for 5 min, followed by 31 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1.2 min, with a final extension step at 72 °C for 5 min. PCR products were analysed on a 1 % (w/v) agarose gel.

RFLP analysis. Using DNAMAN 5.2.2 software, analyses of sequence comparisons, restriction endonuclease selection, restriction maps and unique cutting sites were conducted based on the amplified DNA sequences of the *CAP1* and *GEF1* genes from type strains H99, 125.91, WM626, JEC21, JEC20, WM276, E566, R265, CBS1930, WM161, B4546 and WM779, representing 12 distinct groups of molecular and mating types. Additionally, AD hybrids were analysed using a combination of serotype A (VNI) and serotype D (VNIV) alleles. The restriction endonucleases *Ava*I, *Hgi*CI, *Eco*RII and *Pst*I were chosen and predicted to yield specific RFLP patterns for the differentiation of the distinct groups of all molecular and mating types following digestion of the PCR products from the *CAP1* gene. The PCR products were subjected to separate restriction digestions with *Ava*I and *Hgi*CI enzymes in a first-line identification; PCR products with patterns identical to those of molecular type VNI or VNII (both *MAT*α) were further digested with *Eco*RII, and PCR products with patterns identical to those of molecular type VGIII or VGIV (both *MAT*α) were further digested with *Pst*I. The restriction endonucleases *Eco*T14I and *Hap*II were selected and predicted to yield specific RFLP patterns for the differentiation of the distinct groups of all molecular and mating types following digestion of the PCR products from the *GEF1* gene. The PCR products were subjected to separate restriction digestions with *Eco*T14I and *Hap*II enzymes for identification.

All restriction digestions were performed according to the manufacturer's instructions (TaKaRa). The reaction mixture was incubated at 37 °C for 3 h before separation on a 2.0 % (w/v) agarose gel at 100 V for 1 h and then visualization under UV light.

RESULTS AND DISCUSSION

RFLP identification of the molecular and mating types

The 144 *C. neoformans* and *C. gattii* isolates tested gave positive amplification with both primer pairs: CAP1F/

CAP1R and GEF1F/GEF1R. For all strains assayed, a fragment of approximately 1235 bp was amplified from the genomic DNA with primers CAP1F and CAP1R, and one of approximately 1339 bp with primers GEF1F and GEF1R.

By separate digestions with *Ava*I and *Hgi*CI, RFLP analysis of the fragments amplified from the *CAP1* gene generated 12 different patterns, compatible with the distinct groups of the eight molecular types (VNI, VNII, VNIII, VNIV, VGI, VGII, VGIII and VGIV) and the two mating types (α and a) within the 135 isolates of the *Cryptococcus* species complex. The same restriction pattern was shown by the molecular types VNI and VNII (both *MAT*α) and a different pattern was shown by both VGIII and VGIV (both *MAT*α), as predicted by *in silico* analysis. In concordance with the *in silico* analysis, molecular types VNI and VNII (both *MAT*α) could be differentiated further by *Eco*RII digestion, and VGIII and VGIV (both *MAT*α) could be differentiated further by *Pst*I digestion (Tables 1 and 2; Fig. 2). RFLP analysis of the fragments amplified from the *GEF1* gene via separate digestions with *Eco*T14I and *Hap*II generated 14 different patterns corresponding exactly to the distinct groups of the eight molecular types and the two mating types for the 135 isolates as expected from the *in silico* analysis (Tables 1 and 2; Fig. 3). Thus the 135 cryptococcal isolates were successfully grouped corresponding to their unique molecular and mating types using either of the two PCR-RFLP approaches. No profile variation was observed when the patterns were analysed, confirming the reliability of the two typing approaches. In addition, the unusual AD hybrid of AαDα was also distinguished by the established RFLP patterns by either of the two typing approaches (Tables 1 and 2; Figs 4 and 5).

Identification of VNB strains

The ability of the two RFLP typing approaches to identify the rare genotype VNB and its mating type was also investigated. The VNB *MAT*α strains exhibited the same restriction profile as the molecular type VNI *MAT*α isolates after separate digestions of the *CAP1* gene with *Ava*I, *Hgi*CI and *Eco*RII, whereas the VNB *MAT*a strains showed a unique pattern distinct from the 12 patterns established by separate digestions with *Ava*I and *Hgi*CI (Tables 1 and 2; Fig. 6). The VNB *MAT*α strains displayed a unique pattern in comparison with all patterns established for the *GEF1* gene (Tables 1 and 2; Fig. 5), but the restriction pattern obtained from the VNB *MAT*a strains was identical to the pattern of the molecular type VNI *MAT*a strains. These results suggest that VNB *MAT*α and VNB *MAT*a strains could not both be distinguished from VNI strains by either of the two methods. Therefore, a combination of the two typing approaches is required for the identification of the VNB strains.

Analysis of AD hybrid subtypes

Multilocus genotyping methods such as PCR fingerprinting, amplified fragment length polymorphism analysis and

Table 2. Categories of restriction profiles from the *CAP1* and *GEF1* gene fragments with the corresponding molecular and mating types

Molecular and mating types	RFLP pattern	Profile (<i>CAP1</i>)*				RFLP pattern	Profile (<i>GEF1</i>)*	
		<i>Ava</i> I	<i>Hgi</i> CI	<i>Eco</i> RII	<i>Pst</i> I		<i>Eco</i> T14I	<i>Hap</i> II
VNI <i>MAT</i> α	C1	A1	H1	E1	ND	G1	Ec1	Ha1
VNI <i>MATa</i>	C2	A2	H2	ND	ND	G2	Ec2	Ha2
VNII <i>MAT</i> α	C3	A1	H1	E2	ND	G3	Ec1	Ha3
VNIII <i>MAT</i> α / <i>a</i>	C4	A3	H1	ND	ND	G4	Ec3	Ha4
VNIII <i>MATa</i> / α	C5	A4	H3	ND	ND	G5	Ec4	Ha5
VNIV <i>MAT</i> α	C6	A5	H1	ND	ND	G6	Ec5	Ha6
VNIV <i>MATa</i>	C7	A6	H1	ND	ND	G7	Ec2	Ha6
VGI <i>MAT</i> α	C8	A7	H4	ND	ND	G8	Ec6	Ha7
VGI <i>MATa</i>	C9	A2	H5	ND	ND	G9	Ec7	Ha8
VGII <i>MAT</i> α	C10	A8	H4	ND	ND	G10	Ec5	Ha9
VGII <i>MATa</i>	C11	A2	H6	ND	ND	G11	Ec5	Ha10
VGIII <i>MAT</i> α	C12	A1	H4	ND	P1	G12	Ec2	Ha8
VGIII <i>MATa</i>	C13	A6	H6	ND	ND	G13	Ec8	Ha11
VGIV <i>MAT</i> α	C14	A1	H4	ND	P2	G14	Ec9	Ha8
VNB <i>MAT</i> α	C1	A1	H1	E1	ND	G15	Ec1	Ha8
VNB <i>MATa</i>	C15	A6	H2	ND	ND	G2	Ec2	Ha2
VNIII <i>MAT</i> α / α	C16	A1	H1	E3	ND	G16	Ec10	Ha4

ND, Not done.

*Distinct combination of letters and numbers represents different restriction profiles.

multigene studies (Boekhout *et al.*, 2001; Lengeler *et al.*, 2001; Meyer *et al.*, 2003; Litvinseva *et al.*, 2005a; Bovers *et al.*, 2007, 2008b) have been needed until now for the precise identification of cryptococcal hybrids. However, in the current study, all three mating types (α/a , *a/a* and α/α) of the AD hybrids could be differentiated by either of the two RFLP approaches. They showed distinct restriction patterns from those of the other groups of the molecular and mating types. In spite of the similarity in patterns of *AzD* α and *Az* strains in the RFLP analysis of the *CAP1* gene, an additional band was observed in *AzD* α hybrids with *Eco*RII digestion following gel electrophoresis.

In contrast to the *in silico* analysis in which AD hybrids were analysed using a combination of molecular type VNI

and VNIV alleles, *AaD* α hybrids showed a mixed profile from the genotype VNB *MATa* profile rather than molecular type VNI *MATa* in the RFLP analysis of the *CAP1* gene (Fig. 6). The analysis showed that *AzD* α hybrids displayed partial bands from the VNI *MAT* α profile rather than the VNII *MAT* α profile (Fig. 4), and *AzDa* hybrids exhibited a mixed profile from VNI or VNII (both *MAT* α) and VNIV *MATa* profiles (Fig. 2). In the RFLP analysis of the *GEF1* gene, *AzDa* hybrids exhibited a combination of bands from the patterns of VNI *MAT* α and VNIV *MATa*, *AzD* α hybrids exhibited a combination of bands from the patterns of VNI *MAT* α and VNIV *MAT* α (Fig. 5), and *AaD* α hybrids exhibited a combination of bands from the patterns of VNI or VNB (both *MATa*) and VNIV *MAT* α (Fig. 3).

Fig. 2. RFLP patterns of *CAP1* gene fragments. Fourteen RFLP patterns (C1–C14) were generated with the enzymes *Ava*I (A), *Hgi*CI (H), *Eco*RII (E) and *Pst*I (P). Left panel, RFLP patterns of *C. neoformans*; right panel, RFLP patterns of *C. gattii*. M, Molecular marker.

Fig. 3. RFLP patterns of *GEF1* gene fragments. Fourteen RFLP patterns (G1–G14) were generated with the enzymes *Eco*T14I (E) and *Hap*II (H). Left panel, RFLP patterns of *C. neoformans*; right panel, RFLP patterns of *C. gattii*. M, Molecular marker.

A recent study suggested that many globally recovered AaD α strains originated in sub-Saharan Africa because their *MATa* serotype A alleles were closely related to genotype VNB, which is geographically restricted to sub-Saharan Africa (Litvintseva *et al.*, 2007). Likewise, the RFLP profile generated from the AaD α strains tested here was found to contain bands from genotype VNB but not from molecular type VNI in the RFLP analysis of the *CAP1* gene. The A α Da strains exhibited a mixed profile of VNI *MATa* and VNIV *MATa* in the RFLP analysis of the *GEF1* gene. This hybrid genotype has also been detected by RFLP analysis of the *PLB1* gene and confirmed by phylogenetic analysis in previous studies (Latouche *et al.*, 2003; Litvintseva *et al.*, 2007), suggesting that the predominant distribution of the molecular type VNI may result in the detection of this genotype in many A α Da strains. Similarly, strains of A α D α , a progeny originated from same-sex mating, also had a *MATa* serotype A allele derived from molecular type VNI rather than VNII or VNB in accordance with other research (Litvintseva *et al.*, 2007).

AD hybrids are diploid or aneuploid. Strains with loss of serotype A- or D-specific alleles in some loci, such as *CLA4*, *LAC1*, *URA5* and *IGS1*, have been detected in several

studies (Lengeler *et al.*, 2001; Cogliati *et al.*, 2006; Bovers *et al.*, 2007). Just like the genes at the *MAT* locus, the absence of one mating-type allele was found in a few AD strains (less than 7% in AD hybrids) and a novel AB hybrid (Cogliati *et al.*, 2001; Lengeler *et al.*, 2001; Litvintseva *et al.*, 2005a; Viviani *et al.*, 2006; Bovers *et al.*, 2008b). Because the *MAT* locus is inherited as a single unit (Lengeler *et al.*, 2002), the absence of the *CAP1* or *GEF1* allele in the RFLP analysis indicates loss of the whole *MAT* locus.

In summary, for identification of the eight major molecular types of the *Cryptococcus* species complex, various molecular typing techniques have been developed such as PCR fingerprinting, amplified fragment length polymorphism analysis, RFLP, sequencing and luminex xMAP technology (Boekhout *et al.*, 2001; Meyer *et al.*, 2003; Diaz *et al.*, 2005; Bovers *et al.*, 2007), and the mating types are usually determined by separate PCR analyses or mating assays (Cogliati *et al.*, 2001; Lengeler *et al.*, 2001; Fraser *et al.*, 2003; Campbell *et al.*, 2005). In the present study, the molecular and mating types of all cryptococcal isolates tested were determined simultaneously by RFLP analysis based on either the *CAP1* or the *GEF1* gene. However, the RFLP analysis of the *GEF1* gene seems to be more

Fig. 4. RFLP patterns of *CAP1* gene fragments. Pattern C16 (A α D α) contains the bands from C1 (VNI *MATa*) rather than C3 (VNII *MATa*). A, *Ava*I; H, *Hgi*CI; E, *Eco*RII; M, molecular marker.

Fig. 5. RFLP patterns of *GEF1* gene fragments. Pattern G4 (A α Da) contains the bands from G7 (VNIV *MATa*) and G1 (VNI *MATa*). Pattern G16 (A α D α) contains the bands from G6 (VNIV *MATa*) and G1 (VNI *MATa*). E, *Eco*T14I; H, *Hap*II; M, molecular marker.

Fig. 6. RFLP patterns of *CAP1* gene fragments. Pattern C5 (AaD α) contains the bands from C6 (VNIV MAT α) and C15 (VNB MAT α) rather than C2 (VNI MAT α). A, Aval; H, HgiCI; M, molecular marker.

convenient than the RFLP analysis of the *CAP1* gene and should be a preferred typing method. Overall, the RFLP analysis developed here provides an alternative choice for easy identification of the molecular and mating types of strains belonging to the *Cryptococcus* species complex and may be a useful tool in clinical microbiological and epidemiological studies of this pathogenic yeast.

ACKNOWLEDGEMENTS

We thank Joseph Heitman, John R. Perfect, Anastasia P. Litvintseva, Wiley A. Schell, Sheryl Frank and Anna Floyd (Duke University Medical Center, Durham, USA), Wieland Meyer and Dee Carter (University of Sydney, Sydney, Australia), C. De Vroey (Prince Leopold Institut of Tropical Medicine, Belgium), Maria Anna Viviani (Istituto di Igiene e Medicina Preventiva, Milano, Italy), Kyung J. Kwon-Chung (National Institutes of Health, Bethesda, MD, USA), James W. Kronstad (University of British Columbia, Vancouver, BC, Canada), Teun Boekhout (Centraalbureau voor Schimmelcultures-Fungal Biodiversity Centre, Utrecht, The Netherlands), Jianping Xu (McMaster University, Hamilton, Canada) and Claudete R. Paula (University of São Paulo, São Paulo, Brazil) for strains. We also thank Wieland Meyer for his assistance in correction of the manuscript. This work was supported by a grant (no. 30870108) from the National Natural Science Foundation of China.

REFERENCES

- Barchiesi, F., Cogliati, M., Esposto, M. C., Spreghini, E., Schimizzi, A. M., Wickes, B. L., Scalise, G. & Viviani, M. A. (2005). Comparative analysis of pathogenicity of *Cryptococcus neoformans* serotypes A, D and AD in murine cryptococcosis. *J Infect* 51, 10–16.
- Boekhout, T., Theelen, B., Diaz, M., Fell, J. W., Hop, W. C., Abeln, E. C., Dromer, F. & Meyer, W. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* 147, 891–907.
- Bovers, M., Hagen, F., Kuramae, E. E., Diaz, M. R., Spanjaard, L., Dromer, F., Hoogveld, H. L. & Boekhout, T. (2006). Unique hybrids between the fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*. *FEMS Yeast Res* 6, 599–607.
- Bovers, M., Diaz, M. R., Hagen, F., Spanjaard, L., Duim, B., Visser, C. E., Hoogveld, H. L., Scharringa, J., Hoepelman, I. M. & other authors (2007). Identification of genotypically diverse *Cryptococcus neoformans* and *Cryptococcus gattii* isolates by Luminex xMAP technology. *J Clin Microbiol* 45, 1874–1883.
- Bovers, M., Hagen, F., Kuramae, E. E. & Boekhout, T. (2008a). Six monophyletic lineages identified within *Cryptococcus neoformans* and *Cryptococcus gattii* by multi-locus sequence typing. *Fungal Genet Biol* 45, 400–421.
- Bovers, M., Hagen, F., Kuramae, E. E., Hoogveld, H. L., Dromer, F., St-Germain, G. & Boekhout, T. (2008b). AIDS patient death caused by novel *Cryptococcus neoformans* $\times$ *C. gattii* hybrid. *Emerg Infect Dis* 14, 1105–1108.
- Bui, T., Lin, X., Malik, R., Heitman, J. & Carter, D. (2008). Isolates of *Cryptococcus neoformans* from infected animals reveal genetic exchange in unisexual, α mating type populations. *Eukaryot Cell*, (in press) doi:10.1128/EC.00097-08
- Campbell, L. T., Fraser, J. A., Nichols, C. B., Dietrich, F. S., Carter, D. & Heitman, J. (2005). Clinical and environmental isolates of *Cryptococcus gattii* from Australia that retain sexual fecundity. *Eukaryot Cell* 4, 1410–1419.
- Casadevall, A. & Perfect, J. R. (1998). *Cryptococcus neoformans*. Washington, DC: American Society for Microbiology.
- Cogliati, M., Esposto, M. C., Clarke, D. L., Wickes, B. L. & Viviani, M. A. (2001). Origin of *Cryptococcus neoformans* var. *neoformans* diploid strains. *J Clin Microbiol* 39, 3889–3894.
- Cogliati, M., Esposto, M. C., Tortorano, A. M. & Viviani, M. A. (2006). *Cryptococcus neoformans* population includes hybrid strains homozygous at mating-type locus. *FEMS Yeast Res* 6, 608–613.
- Diaz, M. R., Boekhout, T., Kiesling, T. & Fell, J. W. (2005). Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast *Cryptococcus neoformans*. *FEMS Yeast Res* 5, 1129–1140.
- Fraser, J. A., Subaran, R. L., Nichols, C. B. & Heitman, J. (2003). Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*: implications for an outbreak on Vancouver Island, Canada. *Eukaryot Cell* 2, 1036–1045.
- 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. & other authors (2005). Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature* 437, 1360–1364.
- Keller, S. M., Viviani, M. A., Esposto, M. C., Cogliati, M. & Wickes, B. L. (2003). Molecular and genetic characterization of a serotype A MAT α *Cryptococcus neoformans* isolate. *Microbiology* 149, 131–142.
- Kidd, S. E., Hagen, F., Tschärke, 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.
- Kidd, S. E., Guo, H., Bartlett, K. H., Xu, J. & Kronstad, J. W. (2005). Comparative gene genealogies indicate that two clonal lineages of *Cryptococcus gattii* in British Columbia resemble strains from other geographical areas. *Eukaryot Cell* 4, 1629–1638.

- Kwon-Chung, K. J. & Varma, A. (2006). Do major species concepts support one, two or more species within *Cryptococcus neoformans*? *FEMS Yeast Res* **6**, 574–587.
- 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). Proposal to conserve the name *Cryptococcus gattii* against *C. hondurians* and *C. bacillisporus* (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.
- Lengeler, K. B., Wang, P., Cox, G. M., Perfect, J. R. & Heitman, J. (2000). Identification of the MATa mating-type locus of *Cryptococcus neoformans* reveals a serotype A MATa strain thought to have been extinct. *Proc Natl Acad Sci U S A* **97**, 14455–14460.
- Lengeler, K. B., Cox, G. M. & Heitman, J. (2001). Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. *Infect Immun* **69**, 115–122.
- Lengeler, K. B., Fox, D. S., Fraser, J. A., Allen, A., Forrester, K., Dietrich, F. S. & Heitman, J. (2002). Mating-type locus of *Cryptococcus neoformans*: a step in the evolution of sex chromosomes. *Eukaryot Cell* **1**, 704–718.
- Lin, X., Litvintseva, A. P., Nielsen, K., Patel, S., Floyd, A., Mitchell, T. G. & Heitman, J. (2007). α AD α hybrids of *Cryptococcus neoformans*: evidence of same-sex mating in nature and hybrid fitness. *PLoS Genet* **3**, 1975–1990.
- Litvintseva, A. P., Kestenbaum, L., Vilgalys, R. & Mitchell, T. G. (2005a). Comparative analysis of environmental and clinical populations of *Cryptococcus neoformans*. *J Clin Microbiol* **43**, 556–564.
- Litvintseva, A. P., Thakur, R., Reller, L. B. & Mitchell, T. G. (2005b). Prevalence of clinical isolates of *Cryptococcus gattii* serotype C among patients with AIDS in sub-Saharan Africa. *J Infect Dis* **192**, 888–892.
- Litvintseva, A. P., Thakur, R., Vilgalys, R. & Mitchell, T. G. (2006). Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (serotype A), including a unique population in Botswana. *Genetics* **172**, 2223–2238.
- 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.
- Meyer, W., Castañeda, A., Jackson, S., Huynh, M., Castañeda, E. & the IberoAmerican Cryptococcal Study Group (2003). Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis* **9**, 189–195.
- Mitchell, T. G. & Perfect, J. R. (1995). Cryptococcosis in the era of AIDS – 100 years after the discovery of *Cryptococcus neoformans*. *Clin Microbiol Rev* **8**, 515–548.
- Speed, B. & Dunt, D. (1995). Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans*. *Clin Infect Dis* **21**, 28–36.
- Tortorano, A. M., Viviani, M. A., Rigoni, A. L., Cogliati, M., Roverselli, A. & Pagano, A. (1997). Prevalence of serotype D in *Cryptococcus neoformans* isolates from HIV positive and HIV negative patients in Italy. *Mycoses* **40**, 297–302.
- Trilles, L., Fernández-Torres, B., Lazéra Mdos, S., Wanke, B. & Guarro, J. (2004). *In vitro* antifungal susceptibility of *Cryptococcus gattii*. *J Clin Microbiol* **42**, 4815–4817.
- Viviani, M. A., Cogliati, M., Esposto, M. C., Lemmer, K., Tintelnot, K., Colom Valiente, M. F., Swinne, D., Velegraki, A., Velho, R. & the European Confederation of Medical Mycology (ECMM) Cryptococcosis Working Group (2006). Molecular analysis of 311 *Cryptococcus neoformans* isolates from a 30-month ECMM survey of cryptococcosis in Europe. *FEMS Yeast Res* **6**, 614–619.