Unique hybrids between the fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*

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Abstract

Cryptococcus neoformans and *Cryptococcus gattii* are yeasts that cause meningoencephalitis, but that differ in host range and geographical distribution. *Cryptococcus neoformans* occurs world-wide and mostly infects immunocompromised patients, whereas *C. gattii* occurs mainly in (sub)tropical regions and infects healthy individuals. Anomalous *C. neoformans* strains were isolated from patients. These strains were found to be monokaryotic, and diploid or aneuploid. Amplified Fragment Length Polymorphism (AFLP) and sequence analyses indicated that AFLP genotypes 2 (*C. neoformans*) and 4 (*C. gattii*) were present. The strains were serologically BD. Mating- and serotype-specific PCR reactions showed that the strains were *MAT*a-serotype D/*MAT*α-serotype B. This study is the first to describe naturally occurring hybrids between *C. neoformans* and *C. gattii*.

Introduction

Cryptococcus neoformans and *Cryptococcus gattii* are encapsulated basidiomycetous yeasts that can cause life-threatening meningoencephalitis. *Cryptococcus neoformans* occurs world-wide, whereas *C. gattii* occurs predominantly in tropical and subtropical regions (Kwon-Chung & Bennett, 1984). However, a recent outbreak of *C. gattii* on Vancouver Island, Canada (Kidd *et al.*, 2004), as well as the identification of *C. gattii* isolates in Italy (Montagna *et al.*, 1997), Spain (Baró *et al.*, 1998; Colom *et al.*, 2005) and Greece (Velegraki *et al.*, 2001) has indicated that *C. gattii* may also exist in more temperate climates. The two cryptococcal species also differ in their ability to cause disease: *C. neoformans* is seen as a secondary pathogen because it primarily infects immunocompromised people, whereas the primary pathogen *C. gattii* mainly infects otherwise healthy individuals (Speed & Dunt, 1995; Casadevall & Perfect, 1998; Sorrell, 2001).

Cryptococcus gattii was recently raised from varietal to species level (Kwon-Chung *et al.*, 2002) on the basis of differences from *C. neoformans* seen in DNA fingerprints (Ruma *et al.*, 1996; Sorrell *et al.*, 1996; Meyer *et al.*, 1999;

Boekhout *et al.*, 2001; Meyer *et al.*, 2002; Latouche *et al.*, 2003) and sequence data (Diaz *et al.*, 2000; Xu *et al.*, 2000; Sugita *et al.*, 2001; Chaturvedi *et al.*, 2002; Biswas *et al.*, 2003; Butler & Poulter, 2005). In addition, molecular–genetic analysis of a laboratory cross between *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* showed no evidence of recombination between the two species (Varma *et al.*, 2002).

Cryptococcus neoformans and *C. gattii* can be distinguished by serotype (A, D and AD for the former, B and C for the latter), RAPD pattern (Ruma *et al.*, 1996; Sorrell *et al.*, 1996), PCR fingerprint (Meyer *et al.*, 1999), RFLP pattern (Meyer *et al.*, 2002; Latouche *et al.*, 2003), AFLP fingerprint (Boekhout *et al.*, 2001) and sequencing (Diaz *et al.*, 2000; Xu *et al.*, 2000; Sugita *et al.*, 2001; Chaturvedi *et al.*, 2002; Biswas *et al.*, 2003; Butler & Poulter, 2005; M. Bovers, unpubl. observ.). Within *Cryptococcus neoformans* two varieties have been distinguished, namely var. *grubii* and var. *neoformans*. *Cryptococcus gattii* can be subdivided into four genotypic groups. Table 1 summarizes the relationships between the varieties, serotypes and different genotypic groups.

The existence of serotype AD hybrids, which are hybrids between the two varieties of *C. neoformans*, shows that

Table 1. The subdivision of *Cryptococcus neoformans* and *Cryptococcus gattii* into varieties, serotypes and genotypes

	Serotype	AFLP genotype*	IGS genotype[†]	Molecular genotype[‡]
C. neoformans var. *grubii*	A	1/1A/1B	1A/1B/1C	VNI/VNII
C. neoformans var. *neoformans*	D	2	2A/2B/2C	VNIV
C. neoformans hybrid	AD	3		VNIII
C. gattii	B	4	4A/4B/4C	VGI
C. gattii	B/C	5	5	VGIII
C. gattii	B	6	3	VGII
C. gattii	B/C	7	6	VGIV

*Boekhout *et al.* (2001); [†]Diaz *et al.* (2005); [‡]Meyer *et al.* (2003).

Table 2. Strains used in this study

Strain	Mating-/Serotype	AFLP genotype	Isolated from	Country	Usage*
770616	aDαB	8	Human, CSF	The Netherlands	all
2010404	aDαB	8	Human, CSF	The Netherlands	all
2011225	aDαB	8	Human, CSF	The Netherlands	all
B3501	αD	2	Laboratory strain, genetic offspring of NIH12 × NIH433		F
CBS1622	αB	4	Tumour	Unknown	A, M, S
CBS5467	αD	2	Milk from mastitic cow	Switzerland	M
CBS5757	αB	4	Unknown	Unknown	A
CBS6992	αB	4	Man	USA	C, S
CBS7816	aD	2	Cuckoo dropping	Thailand	M
E566	aB	4	*Eucalyptus camaldulensis*	Australia	M
JEC20	aD	2	Laboratory strain		A, S
JEC21	αD	2	Laboratory strain		A, C, S
WM276	αB	4	Debris of *Eucalyptus tereticornis*	Australia	F

*A, AFLP analysis; C, CGB medium; F, Flow Cytometry; M, mating-serotype PCR; S, Sequence analysis.

somatic fusion and karyogamy occur within *C. neoformans* between strains of different mating-types (*MAT*α and *MAT*a), but the ecological niche where mating occurs has not yet been determined. A hybrid between *C. neoformans* and *C. gattii* has never been found, although there have been predictions that such entities might exist (Chaturvedi *et al.*, 2002; D'Souza *et al.*, 2004). During an investigation of clinical *C. neoformans* isolates from The Netherlands we observed three isolates from two Dutch patients that did not fit the previously defined AFLP genotypes. In this paper we present our analysis of these isolates. Various molecular and conventional techniques indicated that these isolates were hybrids between *C. neoformans* (serotype D, AFLP genotype 2) and *C. gattii* (serotype B, AFLP genotype 4).

Materials and methods

Clinical and reference strains

Three cryptococcal strains were isolated from the cerebrospinal fluid (CSF) of two Dutch patients. Isolate 770616 was isolated in 1977 from a 23-year-old male, who was being treated for an apparent brain tumour and died during treatment. It is not known whether this patient was im-

munocompromised, but he is unlikely to have been HIV-infected because he was treated before the onset of the AIDS epidemic.

Isolates 2010404 and 2011225 were isolated from a 35-year-old male patient in February and May 2001, respectively. In the past, this patient had kept pigeons and it is known that he had visited the Caribbean and Sri Lanka. *Cryptococcus gattii* is known to occur in tropical and subtropical regions. The patient was negative for HIV but had sarcoidosis with resulting low CD4 counts ($0.16 \times 10^9 \, \text{L}^{-1}$). This patient was treated successfully for recurrent cryptococcal meningitis.

All the strains used in this study are listed in Table 2.

Ploidy analysis

Flow cytometry was used to measure the DNA content of the three isolates. Fresh cells were obtained by cultivating yeast cells in 2 mL YPG (1% yeast extract, 1% pepton, 2% D-glucose) medium supplemented with 0.5 M sodium chloride for 24 h at 25 °C, and agitated at 30 r.p.m. The cells were subcultured under the same conditions as described previously, by inoculating 200 μL of this culture into 2 mL YPG medium supplemented with 0.5 M sodium chloride. After

48 h the cells were harvested and quantitatively stained with propidium iodide (Darzynkiewicz *et al.*, 1994). The DNA content of individual cells was determined using a MoFlo™ High-Performance Cell Sorter (Dako Cytomation, Fort Collins, CO). The cell sorter was adjusted with 1.00 μm Fluoresbrite® YG Carboxylate Microspheres and 2.00 μm Fluoresbrite® Polychromatic RED Microspheres (both from Polysciences, Warrington, PA, USA). Forward and side scatter were set at ∼5° and 90°, respectively, the wavelength of the exciting laser beam was 488 nm and fluorescence was measured at 630 nm. A minimum of 10 000 cells were counted and their fluorescence intensities measured. The data were represented as graphs where the *x*-axis represents the fluorescent intensity (logarithmic) and the *y*-axis the amount of measured cells.

Nuclear staining

Strains were grown for 5 days at 24 °C on YPGA medium (1% yeast extract, 1% pepton, 2% D-glucose, 2% technical agar no. 3; Oxoid, Hampshire, UK). A 0.5 μg mL⁻¹ DAPI solution (Sigma-Aldrich, St Louis, MO) in McIlvaine buffer (citric acid 17.6 mM, Na₂HPO₄ 0.16 M, pH 7.0) was used to suspend the cells. They were incubated for 2 h in the DAPI solution before microscopical examination of the stained nuclei (excitation: 358 nm, emission: 461 nm).

Coloration of CGB medium

Strains were grown at 24 °C on CGB medium (Kwon-Chung *et al.*, 1982a). The color of the CGB medium was assessed on days six and fifteen of culturing.

Serology

A Crypto Check serotyping kit from Iatron Laboratories (Tokyo, Japan) was used according to the manufacturer's instructions to test the serotype of the strains.

DNA isolation and AFLP genotyping

Five single colonies from each of the three putative hybrid strains were cultivated for two days at 25 °C under agitation (30 r.p.m.) in liquid YPG medium supplemented with 0.5 M sodium chloride to reduce capsule size. The DNA was isolated according to the method of Bolano *et al.* (2001), with minor modifications.

Yeast cells were incubated for 3–4 h in ureum buffer (urea 8 M, NaCl 0.5 M, Tris 20 mM, EDTA 20 mM, SDS 2% w/v). Phenol–chloroform (1 : 1, pH 8.0) and lysis buffer (0.5% w/v SDS, 0.5% w/v Sarkosyl in TE, pH 7.5) were added 1 : 1 to the pelleted yeast cells and the cells were bead-beated for 3 min at 2500 beats min⁻¹ with sterile sand. After centrifugation the DNA fraction was ethanol-precipitated and dissolved in TE buffer. The AFLP analysis was performed on five single colonies of the putative hybrid strains as described by Boekhout *et al.* (2001).

Sequencing

Sequence reactions were done using primers amplifying portions of Laccase (*LAC*) and Internal Transcribed Spacer (ITS) (Xu *et al.*, 2000), Intergenic Spacer (IGS) (Diaz *et al.*, 2000) and the second largest subunit of RNA polymerase II (*RPB2*) using the bRPB2-6f and bRPB2-7.1r primers described by Matheny (2005). Most of these genetic markers have been used previously to distinguish the different genotypes of *Cryptococcus neoformans* and *C. gattii*. Amplicons were purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NY).

Amplicons from haploid strains were directly sequenced with a BigDye v3.1 Chemistry kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 3700XL DNA analyzer (Applied Biosystems). Amplicons from putative hybrid strains were cloned with a TA Cloning® Kit (Invitrogen, Carlsbad, CA) into *Escherichia coli* DH5α according to the manufacturer's instructions. At least 13 clones were picked randomly, amplified and sequenced.

IGS genotype analysis based on Luminex technology

The putative hybrid strains were analyzed in duplicate using a bead liquid suspension array. The protocol has been described in Diaz & Fell (2005). Specific oligonucleotide probes for each of the six haploid genotypes were used, as well as probes targeting either *C. neoformans* or *C. gattii* (Diaz & Fell, 2005). These probes, which were designed in the Intergenic Spacer region (IGS), were covalently bound to fluorescently color-coded microspheres following the carbodiimide method (Fulton *et al.*, 1997). Biotinylated target amplicons were allowed to anneal with their complementary probe sequences and quantified by addition of the appropiate conjugate, streptavidin R-phycoerythrin. The microspheres were analyzed with a Luminex 100 analyzer (Luminex, Austin, TX), which employs a dual laser system, simultaneously allowing microsphere identity to be determined and the level of conjugate fluorescence to be quantified. Median fluorescent intensity (MFI) values were calculated. A positive signal was defined as twice the background MFI signal, once the background MFI had been subtracted. This assay was done in a multiplex format as described by Diaz & Fell (2005).

Mating–serotype PCR analysis

Several PCRs with mating- and serotype-specific primers were performed on the putative hybrid strains and selected reference strains (Table 2).

Cryptococcus gattii-specific PCR

PCR amplifications were performed in 20 μL volumes containing 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3), 0.1 mM dNTPs, 0.5 U of *Taq* polymerase (Gentaur, Brussels, Belgium), 3 μL of template DNA and 30 pmol of both primers 660 U and 660 L. These primers are based on an anonymous DNA fragment (216 bp) that was amplified by Randomly Amplified Polymorphic DNA (RAPD) analysis from a *C. gattii* strain (Halliday *et al.*, 1999). Amplification conditions were: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min, and a final extension step of 72 °C for 7 min.

Serotype D-specific PCRs

PCR amplifications were performed in 20 μL volumes containing 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3), 0.1 mM dNTPs, 0.5 U of *Taq* polymerase (Gentaur), 2 μL of template DNA and 20 pmol of both primers. The serotype D-specific primer pair JOHE2596/JOHE3240 amplifies a 790-bp region of the *GPA1* gene that is specific for serotype D isolates (Lengeler *et al.*, 2001; Barreto de Oliveira *et al.*, 2004). Amplification conditions were as follows: 96 °C for 5 min, followed by 25 cycles of 96 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and a final extension step of 72 °C for 5 min. Primer pair JOHE7267/JOHE7268 is specific for serotype D *MAT*α isolates and amplifies a 1200-bp fragment of the *STE20*α gene, whereas primer pair JOHE7273/JOHE7275 is specific for serotype D *MAT*a isolates and amplifies an 870-bp fragment of the *STE20*a gene (Barreto de Oliveira *et al.*, 2004). Amplification conditions were 96 °C for 5 min, followed by 30 cycles of 96 °C for 15 s, 66 °C for 15 s and 72 °C for 1 min, with a final extension step of 72 °C for 5 min.

Mating-type-specific PCR

Two mating-type-specific primer sets based on the *STE12* gene were developed using sequences of strains H99 (αA-AFLP1; AF542529), 125.91 (aA-AFLP1; AF542528), JEC20 (aD-AFLP2; AF242352), JEC21 (αD-AFLP2; AF012924), E566 (aB-AFLP4; AY710429), WM276 (αB-AFLP4; AY710430) and CBS6956 (αB-AFLP6; AY421965), and

tested using 13 strains of different genetic background. The primer sequences are listed in Table 3.

STE12αF809/STE12αR1607 amplifies a 760-bp region specific for both *C. neoformans* and *C. gattii MAT*α strains, whereas STE12aF537/STE12aR1299 amplifies a 800-bp region specific for both *C. neoformans* and *C. gattii MAT*a strains. PCR amplifications were performed in 25 μL volumes containing 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3), 0.2 mM dNTPs, 0.5 U of *Taq* polymerase (Gentaur), 1 μL of template DNA and 15 pmol of both primers. Amplification conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min, with a final extension step of 72 °C for 7 min.

Results

Ploidy analysis and karyology

Figure 1 shows the strains B3501 and 770616 as an example. The graph shows the fluorescent intensity of the cells, where the first peak corresponds to cells in the G1 phase and the second peak corresponds to cells in the G2 phase.

The G1 and the G2 peaks of the haploid reference strain B3501 (AFLP genotype 2) were placed at positions 40.7 and 70.7, respectively. The reference strain WM276 (AFLP genotype 4) had a G1 peak at position 34.9 and a G2 peak at position 59.5. The G2 peak of the haploid reference strains coincided with the G1 peaks of 770616, 2010404 and 2011225 (Fig. 1), which were placed at 66.5, 52.1 and 52.3, respectively. The G2 peaks were located at position 125.4, 107.0 and 97.8 for, respectively, strains 770616, 2010404 and 2011225.

The number of nuclei per cell was determined for all putative hybrid strains by DAPI staining. Cells were globose, subglobose to ellipsoidal, and all were monokaryotic (Fig. 2).

Coloration of CGB medium

After 6 days of culturing, CGB medium inoculated with the positive control *Cryptococcus gattii* (CBS1622) was completely blue, while that inoculated with the negative control *Cryptococcus neoformans* (JEC21) remained yellow. The color of the CGB medium of strain 770616 on day 6 was blue-green, but it had turned completely blue on day 15. The CGB medium for strains 2010404 and 2011225 was mostly

Table 3. Primer sequences based on *STE12* for mating-type specific PCR in both *Cryptococcus neoformans* and *Cryptococcus gattii*

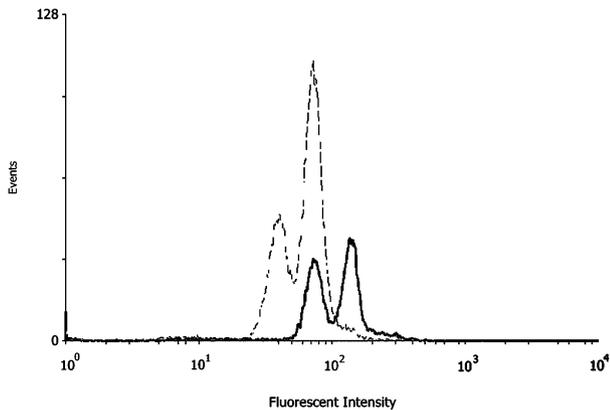| Specificity	Primers	Primer sequence 5′ → 3′
*MAT*α *C. neoformans* + *C. gattii*	STE12α-F809	TTGACCTTTTRTTCCGCAATG
	STE12α-R1607	TTTCTTCTCCCCTGTTTATAGGC
*MAT*a *C. neoformans* + *C. gattii*	STE12a-F537	GTTCTTTGGAATGGCTTATTTCATAT
	STE12a-R1299	GMCTTGCGTGGATCATATCTA

Fig. 1. Analysis of ploidy. The first peak corresponds to the G1 phase and the second to the G2 phase. Haploid strain B3501 is represented by the dashed line and the putative hybrid strain 770616 is represented by the solid line. The G2 peak of B3501 coincides with the G1 peak of 770616. This indicates that 770616 has approximately twice the amount of DNA when compared to B3501. We therefore conclude that 770616 is a diploid.

yellow on day 6, but a slight blue color could be detected. On day 15, the medium of 2010404 and 2011225 was blue-green, while the CGB medium of JEC21 remained yellow (Fig. 3).

Serology

Strains 770616, 2010404 and 2011225 were serotyped with the Crypto Check serotyping kit. All strains agglutinated with both serum factor five (serotype B) and serum factor eight (serotype D) of the kit. This indicates that these strains are serologically BD.

AFLP genotyping

The AFLP patterns (Fig. 4) of the three putative hybrid strains did not match any of the previously defined AFLP genotypes. The AFLP patterns contained specific fragments corresponding to fragments unique to the characteristic pattern of AFLP genotype 2 (serotype D) as well as fragments typical of the AFLP genotype 4 (serotype B). This indicated that genetically the strains combined elements of both AFLP genotypes 2 and 4.

Sequencing

Parts of the Internal Transcribed Spacer (ITS), Intergenic Spacer (IGS), Laccase (*LAC*) and the second largest subunit of RNA polymerase II (*RPB2*) regions were cloned and sequenced. For all three putative BD hybrid strains, 30 clones were sequenced for ITS, IGS and *RPB2*. At least 13 clones were sequenced for *LAC*. The results of the sequence analysis were the same for all three strains.

All 30 ITS clones displayed the sequence corresponding to AFLP genotype 4. No AFLP genotype 2 sequences were detected. In the case of IGS, most of the clones had AFLP genotype 4 sequences; however, some AFLP genotype 2 sequences were also found. Cloning of *RPB2* and *LAC* yielded sequences of both AFLP genotypes. All sequences were submitted to Genbank and have accession numbers DQ286656–DQ286676.
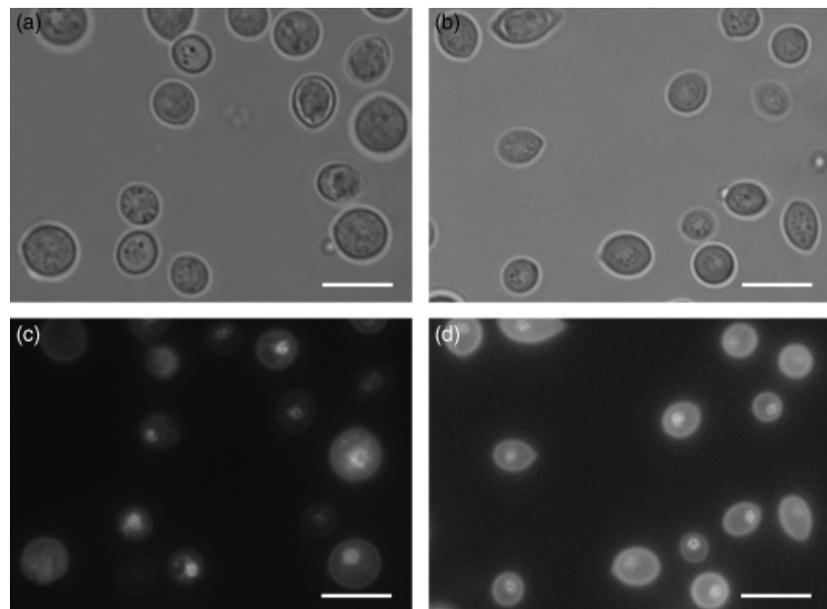

Fig. 2. Cell morphology and nuclear staining by DAPI. Cell morphology of cells of (a) 770616 and (b) 2010404, shows that cells are globose, subglobose to ellipsoidal. DAPI staining of the nucleus in cells of (c) strain 770616 and (d) 2010404 reveals that cells of both strains are monokaryotic. Bar, 10 μm
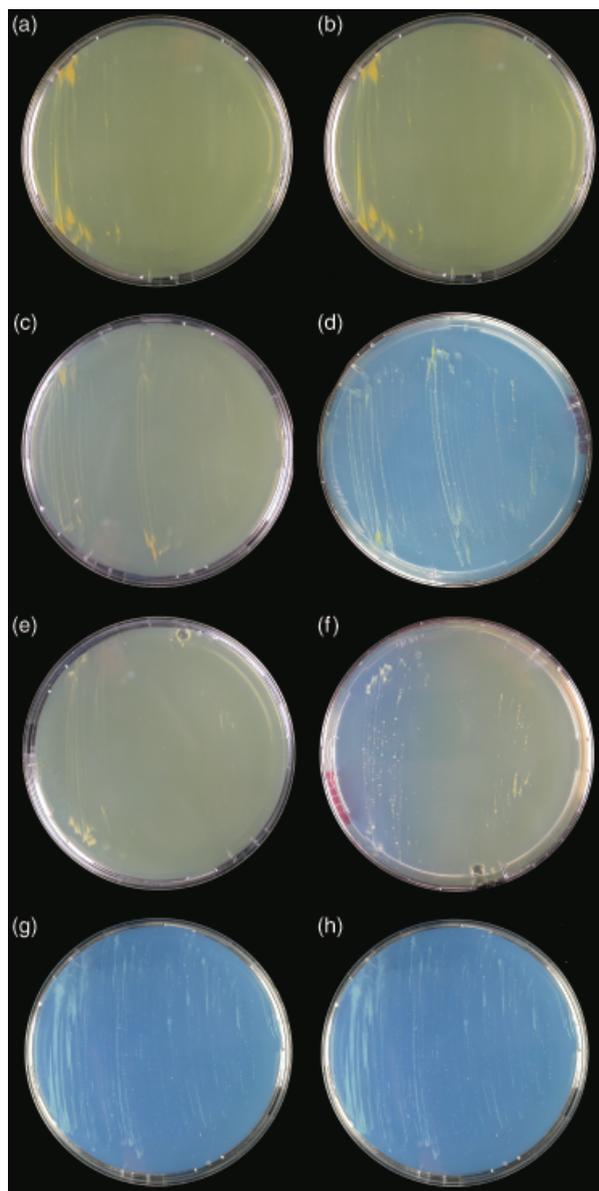
Fig. 3. Coloration of putative BD hybrids and reference strains on CGB medium. Strains were cultured on CGB medium and examined on day 6 (a, c, e, g) and day 15 (b, d, f, h). The medium of JEC21 (*Cryptococcus neoformans* var. *neoformans*) did not turn blue (a, b) whereas the media of both 770616 (c, d) and 2010404 (e, f) were blue-green on day 6 and blue on day 15. The CGB medium had already turned blue on day 6 for *C. gattii* strain CBS6992 (g) and remained so (h). The CGB response for the hybrid strains is intermediate between those of *C. neoformans* and *C. gattii*.

IGS genotype analysis based on Luminex technology

Four specific probes gave a positive signal for all three of the putative hybrid strains. These probes were the *C. neoformans* probe, the *C. gattii* probe, and the probes specific for AFLP genotypes 2 and 4 (Fig. 5).

Mating–serotype PCR analysis

The putative BD hybrid strains were amplified with primers specific for *C. gattii* (primers 660 U/660 L) and *C. neoformans* serotype D (primers JOHE2596/JOHE3240). As expected, the *C. gattii*-specific primers gave amplicons with *C. gattii* strains CBS1622 and E566. Strains CBS5467 and CBS7816, which are both serotype D, yielded PCR products with the serotype D primers (Fig. 6).

The putative BD hybrid strains also yielded amplicons with primer set *STE12*a (primers STE12aF537/STE12aR1299), specific for *MAT*a, and for primer set *STE12*α (primers: STE12αF809/STE12αR1607), which is specific for *MAT*α. In addition, a positive amplification was obtained with the *MAT*a serotype D (primers JOHE7273/JOHE7275) primer set. No amplicon was obtained for the *MAT*α serotype D primer set (primers JOHE7267/JOHE7268). The reference strains gave amplicons with the expected primer pairs. When the *STE12*a primer pair was used, an amplicon was obtained for the *MAT*a strains CBS7816 and E566, whereas the *MAT*α strains CBS1622 and CBS5467 were amplified when the *STE12*α primer pair was used. Amplicons were obtained for CBS7816 with the *MAT*a serotype D primer set and for CBS5467 with the *MAT*α serotype D primer set (Fig. 6). The primer sets *STE12*α and *MF*α (Halliday *et al.*, 1999), which are specific for *MAT*α from *C. gattii*, also produced an amplicon for the putative BD hybrid strains (data not shown).

Discussion

Serotype AD hybrids combining elements of *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *grubii* have been produced in laboratory crosses and have also been isolated from both the environment and from patients. BD hybrids have been generated under laboratory conditions (Kwon-Chung *et al.*, 1982b; Varma *et al.*, 2002), but although there has been some speculation about their existence in nature (Chaturvedi *et al.*, 2002; D'Souza *et al.*, 2004), they had not yet been found.

All our results confirmed that the three isolates from the two Dutch patients are BD hybrids originating from plasmogamy and karyogamy between a *MAT*α serotype B *Cryptococcus gattii* strain of AFLP genotype 4 and a *MAT*a serotype D *C. neoformans* strain of AFLP genotype 2.

The BD hybrids described in this study correspond to 1% of the clinical *C. neoformans* isolates in The Netherlands obtained over the last 27 years, which is an indication that BD hybrids should be found more often than they have been to date. Although a rapid screening with CGB medium (Kwon-Chung *et al.*, 1982a) would miss these hybrids, it is odd that BD hybrids have not been described previously, since the widely used serotyping method is able to distinguish these BD strains.
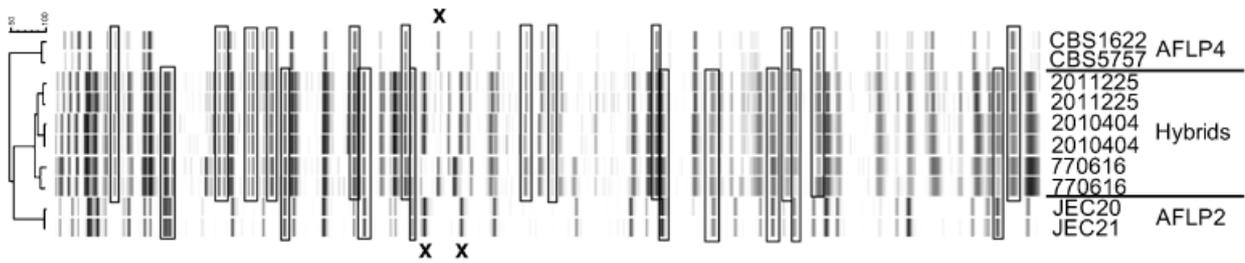
Fig. 4. AFLP fingerprint of the putative BD hybrid strains in duplicate (770616, 2010404 and 2011225) as well as two strains for each of the putative parental AFLP genotype groups (AFLP genotype 4: CBS1622 and CBS5757; AFLP genotype 2: JEC20 and JEC21). Rectangles indicate AFLP fragments occurring in all the putative BD hybrid strains and in one of the putative parental AFLP groups. X indicates AFLP fragments present in some of the putative BD strains and in one of the putative parental AFLP groups. The AFLP fingerprint shows that the putative BD strains contain fragments of both AFLP groups.
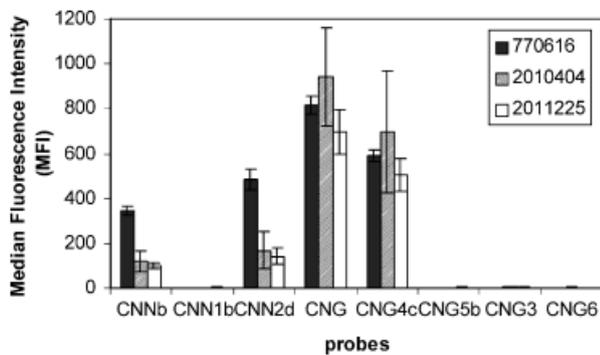
Fig. 5. IGS genotype analysis based on Luminex technology of strains 770616, 2010404 and 2011225. Each strain was analyzed in an assay with eight probes, in duplicate. The background signal was subtracted. CNNc, CNN1b, CNN2d are probes specific for *Cryptococcus neoformans* in general, serotype A and serotype D, respectively. CNG, CNG4c, CNG5b, CNG3, CNG6 correspond, respectively, to the *C. gattii*-specific, AFLP genotype 4-, AFLP genotype 5-, AFLP genotype 6-, and AFLP genotype 7-specific probes. The *C. neoformans* specific probe (CNNb) as well as the serotype D (CNN2d), the *C. gattii* specific probe (CNG) and the AFLP4 specific probe (CNG4c) reacted with the hybrid strains. This shows that the genetic background of AFLP2 and AFLP4 is present in the hybrid strains.

Fig. 6. Results of mating- and serotype-specific PCRs. (a) *Cryptococcus gattii* specific PCR, (b) serotype D specific PCR, (c) *MAT*a specific PCR, (d) *MAT*a serotype D-specific PCR, (e) *MAT*α-specific PCR and (f) *MAT*α serotype D-specific PCR. (m) Marker lane with 1000 bp, 800 bp, 600 bp, 400 bp and 200 bp markers for panels (a–e), but in panel (f): 1200 bp, 1000 bp, 800 bp, 600 bp and 400 bp. Lane 1, 2011225l; lane 2, 2010404; lane 3, 770616; lane 4, CBS5467 (αD); lane 5, CBS7816 (aD); lane 6, CBS1622 (αB); and lane 7, E566 (aB). Reference strain CBS5467 (αD) gave an amplicon when the specific primers for serotype D, *MAT*α and the primers specific for serotype D and *MAT*α were used. Strain CBS7816 (aD) amplified with the serotype D, *MAT*a and the serotype D and *MAT*a specific primers. CBS1622 (αB) gave an amplicon with the *C. gattii*- and the *MAT*α-specific primers, whereas reference strain E566 (aB) amplified with the *C. gattii*- and the *MAT*a-specific primers. The BD hybrid strains gave amplicons when using primers specific for *C. gattii*, serotype D, *MAT*a, *MAT*a and serotype D, and *MAT*α. No amplicon was obtained with primers specific for *MAT*α and serotype D were used. These results indicate that the mating- and serotype composition of the BD hybrids is *MAT*α serotype B and *MAT*a serotype D.

The BD hybrid strains possess two genome copies, and although in all likelihood most of the duplicated genes will eventually be lost, the presence of two copies of one gene present an opportunity for evolving new functions. Within *Saccharomyces cerevisiae*, which arose through whole-genome duplication, it has been shown that some genes have acquired functions differing from those of the corresponding ancestral genes (Kellis *et al.*, 2004). The genus *Saccharomyces* also contains some species that have arisen by hybridization, such as *S. pastorianus* and *S. bayanus* (Vaughan-Martini & Kurtzman, 1985; Nguyen *et al.*, 2000). Therefore, one can speculate that the hybrid between *C. neoformans* and *C. gattii* may eventually evolve into a new species, since postzygotic isolation often occurs in hybrids.

It is interesting to note that BD hybrids contained both AFLP genotype 2 and 4 sequences for *LAC* and *RPB2*, while
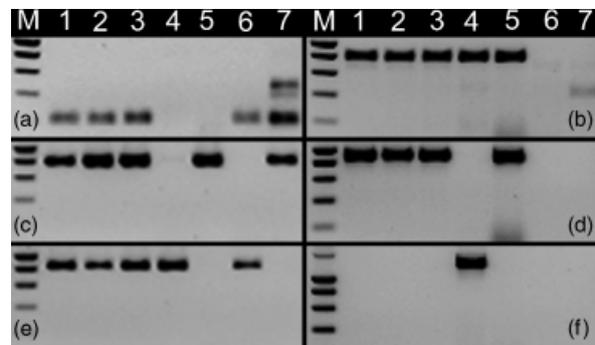
the IGS sequence analysis yielded only a few AFLP genotype 2 clones and ITS analysis exclusively yielded AFLP genotype 4 sequences. This finding may be a chance result, but it may also be a result of concerted evolution, leading to the homogenization of DNA sequences belonging to a repetitive family, in this case rDNA. The occurrence of concerted evolution has been detected in a number of different organisms, e.g. *Escherichia coli*, *Haemophilus influenzae*, *Drosophila melanogaster*, *Xenopus laevis*, *Xenopus mulleri* and *Arabidopsis thaliana* (Liao, 1999).

Two scenarios can be proposed to explain the formation of our diploid hybrid strains. One is somatic fusion followed by karyogamy of a *MAT*α serotype B cell and a *MAT*a serotype D cell. Another is the mating of a *MAT*α serotype B strain and a *MAT*a serotype D strain, giving rise to diploid rather than haploid basidiospores. How our hybrid strains were actually formed remains to be elucidated.

Curiously, all hybrids found in the *C. neoformans* species complex (both AD and BD), have a serotype D parental strain, implying that serotype D strains might be more sexually compatible with other genotypic groups in the *C. neoformans* species complex than the other genotypes.

The existence of BD hybrids is not only scientifically interesting, but may also have implications for human health. A hybrid between *C. neoformans* and *C. gattii* might become a 'superpathogen', that has a world-wide distribution like *C. neoformans*, and is able to infect immunocompetent humans like *C. gattii*, thus combining the malicious characteristics of both species. All of our BD hybrid strains were isolated from patients. This shows that these strains can cause disease, but further investigation is needed to determine whether these strains are distinct in their virulence properties.

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