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Six monophyletic lineages identified within *Cryptococcus neoformans* and *Cryptococcus gattii* by multi-locus sequence typing

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Abstract

Cryptococcus neoformans and *Cryptococcus gattii* are closely related pathogenic basidiomycetous yeasts in which six haploid genotypic groups have been distinguished. The two haploid genotypic groups of *C. neoformans* have been described as variety *grubii* and variety *neoformans*. The four *C. gattii* genotypic groups have, however, not been described as separate taxa. One hundred and seventeen isolates representing all six haploid genotypic groups were selected for multi-locus sequence typing using six loci to investigate if the isolates consistently formed monophyletic lineages. Two monophyletic lineages, corresponding to varieties *grubii* and *neoformans*, were consistently present within *C. neoformans*, supporting the current classification. In addition, four monophyletic lineages corresponding to the previously described genotypic groups were consistently found within *C. gattii*, indicating that these lineages should be considered different taxa as well.

Keywords: *Cryptococcus neoformans*; *Cryptococcus gattii*; Multi-locus sequence typing; Genotype; Haplotype

1. Introduction

Cryptococcus neoformans and *Cryptococcus gattii* are pathogenic basidiomycetous yeasts that belong to the *Filobasidiella* clade of the *Tremellales* (Scorzetti et al., 2002). Although *C. neoformans* and *C. gattii* possess a bipolar mating system with mating-types *MAT*a and *MAT*α (Kwon-Chung, 1975, 1976a), populations of *C. neoformans* and *C. gattii* are predominantly clonal (Brandt et al., 1993, 1996; Chen et al., 1995; Franzot et al., 1997). However, evidence of recombination has been found within subpopulations (Litvintseva et al., 2003, 2005a; Campbell et al., 2005a).

Currently, two varieties are recognized within *C. neoformans*, namely var. *grubii* and var. *neoformans* (Franzot et al., 1999). *C. gattii* has been described as a third variety of *C. neoformans* (Kwon-Chung et al., 1982), but differences in ecology, biochemical and molecular characteristics, as well as the absence of genetic recombination in progeny of crosses between *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* have resulted in the description of *C. gattii* as a separate species (Kwon-Chung et al., 2002). One of the most striking differences between *C. neoformans* and *C. gattii* concerns the host range. Although both species may cause meningoencephalitis, *C. neoformans* mainly causes disease in immunocompromised patients, whereas *C. gattii* may infect otherwise healthy people (Rozenbaum and Goncalves, 1994; Mitchell et al., 1995; Speed and Dunt, 1995; Chen et al., 2000).

A widely used method to differentiate groups within the *C. neoformans–C. gattii* species complex is serotyping (Evans, 1950; Wilson et al., 1968). The separation into different serotypes is based on antigenic differences resulting from variation in capsular polysaccharides (Cherniak and Sundstrom, 1994). Serotype A corresponds to

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C. neoformans var. *grubii*, whereas serotype D corresponds to *C. neoformans* var. *neoformans* (Franzot et al., 1999). *C. gattii* contains isolates of serotypes B and C (Kwon-Chung et al., 2002). Several molecular genotyping methods have been used to distinguish groups within the *C. neoformans–C. gattii* species complex. Six haploid genotypic groups have consistently been found with several molecular fingerprinting methods, such as PCR fingerprinting, RFLP, RAPD and AFLP analysis (Ruma et al., 1996; Ellis et al., 2000; Boekhout et al., 2001; Latouche et al., 2003; Meyer et al., 2003; Kidd et al., 2004), as well as sequence analysis of coding and non-coding regions (Chaturvedi et al., 2002; Biswas et al., 2003; Butler and Poulter, 2005; Diaz et al., 2005). Two of these haploid genotypic groups correspond to the two varieties of *C. neoformans*. The four genotypic groups that can be distinguished within *C. gattii* have, however, not been described as separate taxa. The different haploid genotypic groups and the relationship between variety, serotype and the different genotyping methods are shown in Table 1.

Several sequence analyses have been carried out on *C. neoformans* and *C. gattii* isolates. Many studies studied a single locus (Chaturvedi et al., 2002; Biswas et al., 2003; Butler and Poulter, 2005; Diaz et al., 2005), and the studies that sequenced multiple loci used either *C. neoformans* isolates (Litvintseva et al., 2006) or *C. gattii* isolates (Fraser et al., 2005; Kidd et al., 2005). Two studies used *C. neoformans* as well as *C. gattii* isolates, but only few *C. gattii* isolates were included (Xu et al., 2000) or the *C. gattii* isolates that were studied differed for the various loci (Sugita et al., 2001). Unfortunately, it is difficult to compare genealogies of different genomic regions, which could provide insight into the mode of reproduction, because the sets of isolates differ between studies.

Our multi-locus sequence typing (MLST) study included one hundred and seventeen isolates from clinical and environmental origin that represent all known haploid molecular genotypes of *C. neoformans* and *C. gattii*. Hybrid isolates exist (Tanaka et al., 1999; Cogliati et al., 2000; Boekhout et al., 2001; Lengeler et al., 2001; Bovers et al., 2006; Bovers et al., 2008), but these were not included in our study as the results obtained by analyzing hybrid isolates are expected to be similar to those obtained by analysis of haploid isolates. Six loci including two ribosomal DNA regions, namely Internal Transcribed Spacers 1 and 2 including 5.8S rDNA (ITS) and Intergenic Spacer 1 (IGS1), as well as the laccase gene (*CNLAC1*), the largest and second largest subunit of RNA polymerase II (*RPB1* and *RPB2*) and Translation Elongation Factor 1α (*TEF1*) were selected for our MLST study. MLST analysis of all haploid molecular genotypes present within the *C. neoformans–C. gattii* species complex resulted in the identification of six monophyletic lineages.

2. Materials and methods

2.1. Isolates

One hundred and seventeen haploid isolates of clinical (60%), veterinary (7%), environmental (24%), laboratory (8%) and unknown (1%) origin were used for sequence analyses. Isolates of each of the six haploid genotypic groups currently recognized within the *C. neoformans–C. gattii* species complex were included. The origin, serotype and genotype of the strains are presented in Table 2.

Cryptococcus amylolentus (CBS6039), *Filobasidiella depauperata* (CBS7841) and *Tsuchiyaea wingfieldii* (CBS7118), species which are closely related to *C. neoformans* and

Table 1
Overview of varieties, serotypes and haploid genotypes within *C. neoformans* and *C. gattii*

	Serotype[a]	Amplified fragment length polymorphism (AFLP) genotype[b,c,d]	Molecular genotype[a,e]	IGS genotype[f]	ITS genotype[g]
C. neoformans					
C. neoformans var. grubii	A	1	VNI	1a/1b	1
	A	1A	VNB	1a	1
	A	1B	VNII	1c	1
C. neoformans var. *neoformans*	D	2	VNIV	2a/2b/2c	2
C. gattii					
C. gattii	B/(C)*	4A	VGI	4c	7
	B/(C)*	4B	VGI	4a/4b	3/7
C. gattii	B/C	5A/5C	VGIII	5	5
	B	5B	VGIII	5	5
C. gattii	B/(C)*	6	VGII	3	4
C. gattii	B/C	7	VGIV	6	6

[a] Meyer et al. (2003).
[b] Barreto de Oliveira et al. (2004).
[c] Boekhout et al. (2001).
[d] Kidd et al. (2004).
[e] Litvintseva et al. (2006).
[f] Diaz et al. (2005).
[g] Katsu et al. (2004).
* Serotypes indicated between brackets were not included in our study.

Table 2
AFLP genotype, molecular type, serotype and origin of *C. neoformans* and *C. gattii* isolates

Isolate	Serotype	AFLP genotype[a,b,c]	Molecular type[d,e]	Ploidy	Mating-type	Origin	Reference/ source	ITS	IGS	*RPB1*	*RPB2*	*CNLAC1*	*TEF1*
C. neoformans var. grubii													
125.91 (=CBS10512)	A	1	VNI	nd	a	Cryptococcal meningitis patient, Tanzania	Lengeler et al., 2002	EF211129	EF211249	EF211337	EF211457	EF211577	EF211692
821330	A	1	nd	nd	α	HIV negative female, The Netherlands	L. Spanjaard	EF211130	EF211250	EF211338	EF211458	EF211578	EF211693
930104-I	A	1	nd	nd	α	HIV positive male, The Netherlands	L. Spanjaard	EF211131	EF211251	EF211339	EF211459	EF211579	EF211694
930104-II	A	1	nd	nd	α	HIV positive male, The Netherlands	L. Spanjaard	EF211132	EF211252	EF211340	EF211460	EF211580	EF211695
940441	A	1B	nd	nd	α	HIV positive male, The Netherlands	L. Spanjaard	EF211133	EF211253	EF211341	EF211461	EF211581	EF211696
ATCC90112	A	1	VNI	nd	α	Human, Pennsylvania, USA	Katsu et al., 2004	EF211134	EF211254	EF211342	EF211462	EF211582	EF211697
Bt1	A	1A	VNB	nd	α	AIDS patient, Botswana	Litvintseva et al., 2003	EF211135	EF211255	EF211343	EF211463	EF211583	EF211698
Bt27	A	1A	VNB	nd	α	AIDS patient, Botswana	Litvintseva et al., 2003	EF211136	EF211256	EF211344	EF211464	EF211584	EF211699
Bt63	A	1A	VNB	nd	a	AIDS patient, Botswana	Litvintseva et al., 2003	EF211137	EF211257	EF211345	EF211465	EF211585	EF211700
Bt130	A	1	VNI	nd	a	AIDS patient, Botswana	Litvintseva et al., 2003	EF211138	EF211258	EF211346	EF211466	EF211586	EF211701
Bt206	A	1A	VNB	nd	a	AIDS patient, Botswana	Litvintseva et al., 2003	EF211139	EF211259	EF211347	EF211467	EF211587	EF211702
CBS996[(T)]	A	1	nd	Haploid	α	Blastomycosis from man, type strain of *Candida psicrophylicus*, Argentina	Boekhout et al., 1997	EF211140	EF211260	EF211348	EF211468	EF211588	EF211703
CBS1931	A	1	nd	nd	α	Soil	Boekhout et al., 1997	EF211141	AJ300842*	EF211349	EF211469	EF211589	EF211704
CBS7812	A	1	nd	nd	α	Non-AIDS patient	Boekhout et al., 1997	EF211142	AJ300864*	EF211350	EF211470	EF211590	EF211705
CBS8336	A	1	nd	Haploid	α	Decaying wood of *Cassia* tree, Brazil	Boekhout et al., 2001	EF211143	EF211261	EF211351	EF211471	EF211591	EF211706
CBS8710 (=CBS10515 = H99)	A	1	nd	nd	α	Subculture of type strain of *Cryptococcus neoformans* var. *grubii* (H99)	Boekhout et al., 2001	EF211144	EF211262	EF211352	EF211472	EF211592	EF211707
CBS9172 (=IUM96-2828)	A	1	nd	nd	a	Soil sample in garden of a patient with neighboring birds colonies, rural aviary, Apulia, Italy	CBS	EF211145	EF211263	EF211353	EF211473	EF211593	EF211708
H99 (=CBS8710 = CBS10515)[(T)]	A	1	VNI	Haploid	α	Patient with Hodgkin's disease, type strain of *Cryptococcus neoformans* var. *grubii*, New York, USA	Franzot et al., 1999	EF211146	EF211264	EF211354	EF211474	EF211594	EF211709
H99 5-FOAr	A	1	nd	nd	α	5-Fluoroorotic acid (5-FOA)-resistant mutant of strain H99	Lengeler et al., 2001	EF211147	EF211265	EF211355	EF211475	EF211595	EF211710
Hamden C3′1	A	1A	nd	Haploid	α	Pigeon dropping, Belo Horizonte, Brazil	Katsu et al., 2004	EF211148	EF211266	EF211356	EF211476	EF211596	EF211711
ICB23 (=MT23)	A	1	nd	nd	α	Non-AIDS, Sao Paulo, Brazil	Barreto de Oliveira et al., 2004	EF211149	EF211267	EF211357	EF211477	EF211597	EF211712
ICB87 (=MT87)	A	1	nd	nd	α	Non-AIDS, Sao Paulo, Brazil	Barreto de Oliveira et al., 2004	EF211150	EF211268	EF211358	EF211478	EF211598	EF211713
ICB89 (=YT6)	A	1	nd	nd	α	AIDS patient, Natal, Brazil	Barreto de Oliveira et al., 2004	EF211151	EF211269	EF211359	EF211479	EF211599	EF211714
ICB154 (=MT154)	A	1A	nd	Diploid	α	Non-AIDS, Sao Paulo, Brazil	Barreto de Oliveira et al., 2004	EF211152	EF211270	EF211360	EF211480	EF211600	EF211715
ICB165 (=MTB16)	A	1A/1B	nd	Diploid	α	Pigeon dropping, Rio de Janeiro, Brazil	Barreto de Oliveira et al., 2004	EF211153	EF211271	EF211361	EF211481	EF211601	EF211716
ICB166 (=MTB18)	A	1B	nd	nd	α	Pigeon dropping, Rio de Janeiro, Brazil	Barreto de Oliveira et al., 2004	EF211154	EF211272	EF211362	EF211482	EF211602	EF211717
ICB175 (=MTW3)	A	1B	nd	nd	α	Pigeon dropping, Sao Paulo, Brazil	Barreto de Oliveira et al., 2004	EF211155	EF211273	EF211363	EF211483	EF211603	EF211718

ICB176 (=MTW5)	A	1	nd	nd	α	Pigeon dropping, Sao Paulo, Brazil	Barreto de Oliveira et al., 2004	EF211156	EF211274	EF211364	EF211484	EF211604	EF211719
ICB178 (=MTW9)	A	1A/1B	nd	Diploid	α	Pigeon dropping, Sao Paulo, Brazil	Barreto de Oliveira et al., 2004	EF211157	EF211275	EF211365	EF211485	EF211605	EF211720
ICB186 (=MTPI4)	A	1	nd	nd	α	Hollow trees, Piaui, Brazil	Barreto de Oliveira et al., 2004	EF211158	EF211276	EF211366	EF211486	EF211606	EF211721
ICB187 (=MTPI7)	A	1	nd	nd	α	Hollow trees, Piaui, Brazil	Barreto de Oliveira et al., 2004	EF211159	EF211277	EF211367	EF211487	EF211607	EF211722
ICB188 (=MTPI9)	A	1	nd	nd	α	Hollow trees, Piaui, Brazil	Barreto de Oliveira et al., 2004	EF211160	EF211278	EF211368	EF211488	EF211608	EF211723
M27049	A	1	VNI	nd	α	Clinical, Johannesburg, South Africa	W. Meyer	EF211161	EF211279	EF211369	EF211489	EF211609	EF211724
NIH 296	A	1	nd	nd	α	Non-AIDS patient, USA	Boekhout et al., 2001	EF211162	EF211280	EF211370	EF211490	EF211610	EF211725
P152	A	1	nd	nd	α	AIDS patient, Zimbabwe	Boekhout et al., 2001	EF211163	EF211281	EF211371	EF211491	EF211611	EF211726
PAH-21b	A	1B	nd	nd	α	HIV negative patient, asthma, steroids, Australia	D'Souza et al., 2004	EF211164	EF211282	EF211372	EF211492	EF211612	EF211727
PAH-22	A	1B	nd	nd	α	HIV negative patient, systemic lupus erythematosus, steroids, Australia	D'Souza et al., 2004	EF211165	EF211283	EF211373	EF211493	EF211613	EF211728
RDA 1335 AvB0	A	1	nd	Diploid	α	AIDS patient, The Netherlands	Diaz et al., 2000	EF211166	AJ300883*	EF211374	EF211494	EF211614	EF211729
RV64610	A	1A	nd	Haploid	α	AIDS patient, Rwanda	Diaz et al., 2000	EF211167	AJ300862*	EF211375	EF211495	EF211615	EF211730
RV65662	A	1A	nd	Haploid	α	AIDS patient, Portugal (visited Venezuela)	Diaz et al., 2000	EF211168	AJ300861*	EF211376	EF211496	EF211616	EF211731
TN/ENV/2 (=WM721)	A	1	VNI	nd	α	Pigeon dropping, Madras, India	W. Meyer	EF211169	EF211284	EF211377	EF211497	EF211617	EF211732
UON 11536	A	1B	VNII	nd	α	HIV positive human, Kwa Mashu, South Africa	W. Meyer	EF211170	EF211285	EF211378	EF211498	EF211618	EF211733
WM 02.33 (=CDCR267)	A	1	nd	nd	α	Canada	W. Meyer	EF211171	EF211286	EF211379	EF211499	EF211619	EF211734
WM148 (=CBS10085)[R]	A	1	VNI	Haploid	α	HIV positive human, reference strain of molecular type VNI, Sydney, Australia	Meyer et al., 1999	EF211172	EF211287	EF211380	EF211500	EF211620	EF211735
WM626 (=CBS10084)[R]	A	1B	VNII	nd	α	Immunocompetent human, reference strain of molecular type VNII, Sydney, Australia	Meyer et al., 1999	EF211173	EF211288	EF211381	EF211501	EF211621	EF211736
WM714	A	1B	nd	Diploid	α	Cat paranasal, Australia	Boekhout et al., 2001	EF211174	EF211289	EF211382	EF211502	EF211622	EF211737
C. neoformans var. *neoformans*													
B-3501 (=CBS6900)	D	2	VNIV	Haploid	α	Genetic offspring of CBS6885xCBS7000 (=NIH12 x NIH433)	Boekhout et al., 2001	EF211175	EF211290	EF211383	EF211503	EF211623	EF211738
BD5	D	2	nd	Diploid	α	AIDS patient, France	Boekhout et al., 2001	EF211176	EF211291	EF211384	EF211504	EF211624	EF211739
CBS882[T]	D	2	nd	nd	α	Nasal tumour of horse, type strain of *Torula nasalis*, USA	Boekhout et al., 1997	EF211177	AJ300890*	EF211385	EF211505	EF211625	EF211740
CBS918	D	2	nd	nd	α	Dead white mouse, the Netherlands	Boekhout et al., 1997	EF211178	AJ300917*	EF211386	EF211506	EF211626	EF211741
CBS5467	D	2	nd	Haploid	α	Milk from mastitic cow, Switzerland	Boekhout et al., 1997	EF211179	AJ300912*	EF211387	EF211507	EF211627	EF211742
CBS5728	D	2	nd	Haploid	α	Non-meningitic cellulitis & osteomyelitis, USA	Boekhout et al., 1997	EF211180	AJ300906*	EF211388	EF211508	EF211628	EF211743
CBS6885 (=NIH 12)	D	2	nd	nd	α	Man, lesion on bone, USA	Boekhout et al., 1997	EF211181	AJ300901*	EF211389	EF211509	EF211629	EF211744
CBS6886 (=NIH 430)	D	2	nd	Haploid	α	Dropping of pigeon	Boekhout et al., 1997	EF211182	AJ300886*	EF211390	EF211510	EF211630	EF211745
CBS6995	D	2	nd	Haploid	a	Non-AIDS patient, Illinois, USA	Boekhout et al., 1997	EF211183	AJ300897*	EF211391	EF211511	EF211631	EF211746
CBS7816	D	2	nd	nd	a	Cuckoo dropping, Thailand	Boekhout et al., 1997	EF211184	AJ300900*	EF211392	EF211512	EF211632	EF211747
ICB105 (=MT105)	D	2	nd	nd	α	Non-AIDS, Sao Paulo, Brazil	Barreto de Oliveira et al., 2004	EF211185	EF211292	EF211393	EF211513	EF211633	EF211748

(*continued on next page*)

Table 2 (*continued*)

Isolate	Serotype	AFLP genotype[a,b,c]	Molecular type[d,e]	Ploidy	Mating-type	Origin	Reference/ source	ITS	IGS	*RPB1*	*RPB2*	*CNLAC1*	*TEF1*
ICB106 (=MT106)	D	2	nd	nd	α	Non-AIDS, Sao Paulo, Brazil	Barreto de Oliveira et al., 2004	EF211186	EF211293	EF211394	EF211514	EF211634	EF211749
ICB109 (=MT109)	D	2	nd	nd	α	Non-AIDS, Sao Paulo, Brazil	Barreto de Oliveira et al., 2004	EF211187	EF211294	EF211395	EF211515	EF211635	EF211750
ICB163 (=MT163)	D	2	nd	nd	α	Cerebrospinal fluid, USA	Barreto de Oliveira et al., 2004	EF211188	EF211295	EF211396	EF211516	EF211636	EF211751
JEC20 (=CBS10511 = NIH-B4476)	D	2	nd	Haploid	a	Congenic pair with JEC21 that differs only in matingtype	Kwon-Chung et al., 1992a	EF211189	EF211296	EF211397	EF211517	EF211637	EF211752
JEC21 (=CBS10513 = NIH-B4500)	D	2	nd	Haploid	α	Congenic pair with JEC20 that differs only in matingtype	Kwon-Chung et al., 1992a	EF211190	EF211297	EF211398	EF211518	EF211638	EF211753
JEC171	D	2	nd	nd	a	Serotype D, MATa *ade2 lys2;* derived from JEC20/JEC21	Lengeler et al., 2001	EF211191	EF211298	EF211399	EF211519	EF211639	EF211754
MT-B12	D	2	nd	nd	α	Pigeon droppings, Sao Paulo, Brazil	M.S. Lazera	EF211192	EF211299	EF211400	EF211520	EF211640	EF211755
RKI-M318/90	D	2	VNIV	nd	α	Human, Germany	Katsu et al., 2004	EF211193	EF211300	EF211401	EF211521	EF211641	EF211756
WM629 (=CBS10079)[(R)]	D	2	VNIV	Haploid	α	HIV positive human, reference strain of molecular type VNIV, Melbourne, Australia,	Meyer et al., 1999	EF211194	EF211301	EF211402	EF211522	EF211642	EF211757
C. gattii AFLP4 = VGI													
48A	B	4A	nd	nd	α	Lung of a goat, Spain	Diaz et al., 2000	EF211195	AJ300934*	EF211403	EF211523	EF211643	EF211758
503 2738 (=WM1251)	B	4	VGI	nd	α	Human, Papua New Guinea	Katsu et al., 2004	EF211196	EF211302	EF211404	EF211524	EF211644	EF211759
56A	B	4A	nd	Haploid	α	Gut of a goat, Spain	Diaz et al., 2000	EF211197	AJ300932*	EF211405	EF211525	EF211645	EF211760
CBS883[(T)]	B	4B	nd	nd	α	Infected skin, syntype *Cryptococcus hondurianus*, Honduras	Boekhout et al., 1997	EF211198	EF211303	EF211406	EF211526	EF211646	EF211761
CBS919[(T)]	B	4B	nd	Haploid	α	Meningoencephalic lesion, type strain of *Torulopsis neoformans* var. *sheppei*, USA	Boekhout et al., 1997	EF211199	AJ300928*	EF211407	EF211527	EF211647	EF211762
CBS1622	B	4B	nd	nd	α	Man, Tumour, Lille, France	Boekhout et al., 1997	EF102028	EF102032	EF102061	EF102051	EF102071	EF102047
CBS6289 (=CBS8273 = RV20186 = NIH B-3939)	B	4A	VGI	nd	a[g]	Subculture of type strain of *Cryptococcus gattii* (RV 20186)	Boekhout et al., 1997	EF211200	AJ300937*	EF211408	EF211528	EF211648	EF211763
CBS6290	B	4A	nd	Haploid	α	Man, Congo (Zaire)	Boekhout et al., 1997	EF211201	AJ300930*	EF211409	EF211529	EF211649	EF211764
CBS6992 (=NIH 17)	B	4B	nd	Haploid	α	Man, USA	Boekhout et al., 1997	EF102029					
AJ300923*EF102033		EF102062	EF102052			EF102072	EF102048						
CBS6998 (=NIH 365)	B	4	nd	nd	a[g]	Human, Thailand	Boekhout et al., 1997	EF211202	AJ300925*	EF211410	EF211530	EF211650	EF211765
CBS7229[(T)]	B	4B	nd	nd	a	Meningitis, type strain *Cryptococcus neoformans* var. *shanghaiensis*, China	Boekhout et al., 1997	EF211203	AJ300926*	EF211411	EF211531	EF211651	EF211766
CBS7748 (=IFM50902)	B	4B	VGI	nd	α	Air in *Eucalyptus camaldulensis* hollow, Balranald, South Australia, Australia	Boekhout et al., 1997	EF211204	AJ300927*	EF211412	EF211532	EF211652	EF211767
CBS8273 (=CBS6289 = RV20186 = NIH B-3939)	B	4	VGI	nd	a[g]	Subculture of type strain of *Cryptococcus gattii* (RV 20186)	Boekhout et al., 1997	EF211205	EF211304	EF211413	EF211533	EF211653	EF211768
E566	B	4	VGI	nd	a[f]	*E. camaldulensis* tree #19 hollow 4, Renmark Australia	Halliday, 2000	EF211206	EF211305	EF211414	EF211534	EF211654	EF211769
RV20186 (=CBS6289 = CBS8273 = NIH B-3939)[(T)]	B	4A	VGI	nd	a[g]	Cerebrospinal fluid, type strain of *Cryptococcus gattii*, Congo (Zaire)	Gatti and Eeckels, 1970	EF211207	EF211306	EF211415	EF211535	EF211655	EF211770
RV54130	B	4B	nd	Haploid	a	Second isolate of *C. neoformans* var. *shanghaiensis*, China	Boekhout et al., 2001	EF211208	EF211307	EF211416	EF211536	EF211656	EF211771

WM176	B	4B	nd	nd	α	*Eucalyptus citriodora*, USA	Boekhout et al., 2001	EF211209	EF211308	EF211417	EF211537	EF211657	EF211772
WM179 (=CBS10078)[R]	B	4	VGI	Haploid	α	Immunocompetent human, reference strain of molecular type VGI, Sydney, Australia	Meyer et al., 2003	EF211210	EF211309	EF211418	EF211538	EF211658	EF211773
WM276 (=CBS10510)	B	4	VGI	Haploid	α	*Eucalyptus tereticornis*, Mt. Annan, New South Wales, Australia	Kidd et al., 2005	EF211211	EF211310	EF211419	EF211539	EF211659	EF211774
WM830	B	4	VGI	nd	a	Immunocompetent, Human, Papua New Guinea	Katsu et al., 2004	EF211212	EF211311	EF211420	EF211540	EF211660	EF211775
C. gattii AFLP5 = VGIII													
380C	C	5C	nd	nd	α	Unknown	Boekhout et al., 2001	EF211213	EF211312	EF211421	EF211541	EF211661	EF211776
384C	C	5C	nd	nd	α	Patient, USA	Boekhout et al., 2001	EF211214	EF211313	EF211422	EF211542	EF211662	EF211777
CBS5758	C	5C	nd	nd	α	Unknown	Boekhout et al., 1997	EF211215	AJ300929*	EF211423	EF211543	EF211663	EF211778
CBS6955[T] (=NIH 191 = ATCC32608)	C	5C	VGIII	nd	a[f, g]	Human, type strain of *Cryptococcus bacillisporus*, California, USA	Boekhout et al., 1997	EF211216	AJ300940*	EF211424	EF211544	EF211664	EF211779
CBS6993 (=NIH 18)	C	5C	VGIII	Haploid	α	Human, California, USA	Boekhout et al., 1997	EF211217	EF211314	EF211425	EF211545	EF211665	EF211780
CBS6996	B	5C	nd	nd	α	Man	Boekhout et al., 1997	EF211218	AJ300939*	EF211426	EF211546	EF211666	EF211781
CBS8755 (=HOO58-I-682)	C	5A	nd	Haploid	α	Detritus of almond tree, Colombia	Boekhout et al., 2001	EF211219	EF211315	EF211427	EF211547	EF211667	EF211782
CN043	B	5	VGIII	nd	α	Human, Auckland, New Zealand	Katsu et al., 2004	EF211220	EF211316	EF211428	EF211548	EF211668	EF211783
WM161 (=CBS10081)[R]	B	5B	VGIII	nd	α	*Eucalyptus camaldulensis* wood from hollow, reference strain of molecular type VGIII, San Diego, USA	Meyer et al., 2003	EF211221	EF211317	EF211429	EF211549	EF211669	EF211784
WM726	B	5B	VGIII	nd	α	*Eucalyptus citriodora*, San Diego, USA	Boekhout et al., 2001	EF211222	EF211318	EF211430	EF211550	EF211670	EF211785
WM728	B	5B	VGIII	Haploid	α	*Eucalyptus* sp. debris from car park of zoo, San Diego, USA	Boekhout et al., 2001	EF211223	EF211319	EF211431	EF211551	EF211671	EF211786
C. gattii AFLP6 = VGII													
A1M F2866	B	6	VGII	nd	α	Dead wild Dall's porpoise lymph node, Shores of Gulf Island, Canada	Kidd et al., 2004	EF211224	EF211320	EF211432	EF211552	EF211672	EF211787
A1M F2932	B	6	VGII	nd	α	Immunocompetent male, lung, Kelowna, Canada	Kidd et al., 2004	EF211225	EF211321	EF211433	EF211553	EF211673	EF211788
A1M R265 (=CBS10514)	B	6	VGII	Haploid	α	Immunocompetent male, Duncan, Vancouver Island, Canada	Kidd et al., 2004	EF211226	EF211322	EF211434	EF211554	EF211674	EF211789
A1M R269	B	6	VGII	nd	α	Immunocompetent female, Victoria, Canada	Kidd et al., 2004	EF211227	EF211323	EF211435	EF211555	EF211675	EF211790
A1M R271	B	6	VGII	Haploid	α	Immunocompetent male, Nanoose Bay, Vancouver Island, Canada	Kidd et al., 2004	EF211228	EF211324	EF211436	EF211556	EF211676	EF211791
A1M R368	B	6	VGII	nd	α	Immunocompetent male, Victoria, Canada	Kidd et al., 2004	EF211229	EF211325	EF211437	EF211557	EF211677	EF211792
A1M R406	B	6	VGII	Haploid	α	Immunocompetent female, Nanaimo, Vancouver Island, Canada	Kidd et al., 2004	EF211230	EF211326	EF211438	EF211558	EF211678	EF211793
A1M R409	B	6	VGII	Haploid	α	Immunocompetent female, Victoria, Canada	Kidd et al., 2004	EF211231	EF211327	EF211439	EF211559	EF211679	EF211794
CBS1930	B	6	VGII	Haploid	a[h]	Sick goat, Aruba	Boekhout et al., 1997	EF211232	AJ300919*	EF211440	EF211560	EF211680	EF211795
CBS6956 (=NIH 444 = ATCC32609)	B	6	VGII	Haploid	α	Immunocompetent human, Seattle, USA,	Boekhout et al., 1997	EF211233	AJ300920*	EF211441	EF211561	EF211681	EF211796

(*continued on next page*)

Table 2 (*continued*)

Isolate	Serotype	AFLP genotype[a, b, c]	Molecular type[d, e]	Ploidy	Mating-type	Origin	Reference/ source	ITS	IGS	*RPB1*	*RPB2*	*CNLAC1*	*TEF1*
CBS7750	B	6	VGII	Haploid	α	*Eucalyptus camaldulensis* bark debris, San Francisco, USA	Boekhout et al., 1997	EF211234	AJ300922[*]	EF211442	EF211562	EF211682	EF211797
CBS8684	B	6	VGII	Haploid	α	Nest of wasp, Uruguay	Boekhout et al., 2001	EF211235	EF211328	EF211443	EF211563	EF211683	EF211798
HEC11102	B	6	VGII	nd	a[h]	Human, Rio de Janeiro, Brazil	Katsu et al., 2004	EF211236	EF211329	EF211444	EF211564	EF211684	EF211799
ICB184 (=MTPI2)	B	6	VGII	Haploid	α	Hollow trees, Piaui, Brazil	Barreto de Oliveira et al., 2004	EF211237	EF211330	EF211445	EF211565	EF211685	EF211800
RAM2	B	6	VGII	nd	α	*Eucalyptus camaldulensis*, Arnhemland, Northern Territory, Australia	Katsu et al., 2004	EF211238	EF211331	EF211446	EF211566	EF211686	EF211801
WM178 (=IFM 50894 = CBS10082)[(R)]	B	6	VGII	Haploid	α	Immunocompetent human, lung, reference strain of molecular type VGII, Sydney, Australia	Meyer et al., 2003	EF211239	EF211332	EF211447	EF211567	EF211687	EF211802
C. gattii AFLP7 = VGIV													
B-5742	C	7	VGIV	nd	α[f]	Human, Punjab, India	Katsu et al., 2004	EF211240	EF211333	EF211448	EF211568	EF211688	EF211803
B5748	B	7	VGIV	nd	α	HIV positive patient, India	Diaz and Fell, 2005	EF211241	EF211334	EF211449	EF211569	EF211689	EF211804
M27055	C	7	VGIV	nd	α	Clinical, Johannesburg, South Africa	Latouche et al., 2002	EF211242	EF211335	EF211450	EF211570	EF211690	EF211805
WM779 (=IFM50896 = CBS10101)[(R)]	C	7	VGIV	Haploid	α	Cheetah, reference strain of molecular type VGIV, Johannesburg, South Africa	Meyer et al., 2003	EF211243	EF211336	EF211451	EF211571	EF211691	EF211806

GenBank accession numbers of all sequenced regions are indicated.
nd, not determined.
[a] Boekhout et al. (2001).
[b] Barreto de Oliveira et al. (2004).
[c] F. Hagen (unpublished data).
[d] Meyer et al. (2003).
[e] Litvintseva et al. (2006).
[f] Fraser et al. (2005).
[g] CBS List of Cultures.
[h] Confirmed by *MAT*a *STE12* primers (Bovers et al., 2006).
[*] Diaz et al., 2000).
[(R)] Reference strain (Meyer et al., 2003).
[(T)] Type strain.

C. gattii (Fell et al., 1992; Mitchell et al., 1992; Guého et al., 1993; Kwon-Chung et al., 1995; Fell et al., 2000; Scorzetti et al., 2002), were included as outgroup species. *Cryptococcus podzolicus* (CBS6819 and CBS7717) was added to the outgroup, because this species is, as far as we are aware of, the only other heterobasidiomycetous yeast in which the *CNLAC1* gene that expresses laccase in *C. neoformans* and *C. gattii* is present (Petter et al., 2001). The origin of the outgroup strains is described in Table 3.

2.2. Ploidy analysis

The ploidy of a subset of isolates ($N = 43$) was determined by flow cytometry. The subset represented all six haploid genotypic groups currently recognized within the *C. neoformans–C. gattii* species complex. The analysis was carried out as described in Bovers et al. (2006). Briefly, cells were grown in YPG (1% yeast extract, 1% pepton, 2% D-glucose) supplemented with 0.5 M sodium chloride. Propidium iodide was used to quantitatively stain the DNA present in the cells (Darzynkiewicz et al., 1994) and a MoFlo™ High-Performance Cell Sorter (Dako Cytomation, Fort Collins, CO) was used to measure the fluorescence of at least 20,000 individual cells. The wavelength of the exciting laser beam was 488 nm and fluorescence was measured at 630 nm. The data was represented as a graph where the *x*-axis represents the fluorescence intensity (logarithmic) and the *y*-axis represents the amount of measured cells. The ploidy of the isolates was determined by comparison of the results with haploid reference isolates H99, B3501, WM276 and A1M-R265.

2.3. Mating-type and serotype analyses

The mating-type was determined for all isolates included in the study. The serotype A (JOHE2596/JOHE3241) and serotype D (JOHE2596/JOHE3240) specific primer sets (Lengeler et al., 2001) were used to determine the serotype of *C. neoformans* isolates. In addition, the mating-type of serotype A isolates was determined by PCR amplification with the *MAT*a-serotype A (JOHE7270/JOHE7272) and the *MAT*α-serotype A (JOHE7264/JOHE7265) specific primer sets (Barreto de Oliveira et al., 2004), whereas the mating-type of serotype D isolates was determined by PCR amplification with the *MAT*a-serotype D (JOHE7273/JOHE7275) and the *MAT*α-serotype D (JOHE7267/JOHE7268) specific primer sets (Barreto de Oliveira et al., 2004).

The mating-type of *C. gattii* isolates was determined by amplification with the *MAT*α specific primer sets MFαU/MFαL and STE12αU/STE12αL (Halliday et al., 1999). *MAT*a was assigned to those *C. gattii* isolates that failed to amplify with both of the *MAT*α specific primer sets as was carried out previously by Campbell et al. (2005b). In some cases additional verification was performed using MATa specific *STE12* primers (Bovers et al., 2006).

All PCR amplifications were carried out in a total volume of 20 μl containing 0.1 mM dNTPs, 0.5 U of *Taq* polymerase (Gentaur, Brussels, Belgium), 1–3 μl of template DNA and varying amounts of primers. Primer sequences, buffer composition, amount of added primers and amplification conditions are listed in Table 4.

2.4. Selected loci and their chromosomal location

Six loci were included in our MLST analysis. Chromosomal locations were based on the *C. neoformans* serotype D complete genome of isolate JEC21 (Loftus et al., 2005). Two ribosomal DNA regions, located on chromosome 2, were included, namely Internal Transcribed Spacers 1 and 2 including 5.8S rDNA (ITS) and Intergenic Spacer 1 (IGS1). Ribosomal DNA regions are often used in phylogenetic analyses and have been used to study *C. neoformans* and *C. gattii* (Xu et al., 2000; Diaz et al., 2000, 2005; Litvintseva et al., 2003, 2006; Katsu et al., 2004; Fraser et al., 2005). Furthermore, three loci were included that had been selected for the Assembling the Fungal Tree of Life (AFTOL) project (James et al., 2006). These regions are the largest subunit of RNA polymerase II (*RPB1*)

Table 3
Origin of outgroup isolates used in ITS, *RPB1* and *RPB2* analyses

	Isolate	Origin	Reference	ITS	*RPB1*	*RPB2*
Cryptococcus amylolentus	CBS6039[a]	Frass of larvae of *Enneadesmus forficulus* (buprestid beetle) in *Dombeya rotundifolia*, type strain of *Cryptococcus amylolentus* (=*Candida amylolenta*), South Africa	CBS	EF211244	EF211452	EF211572
Cryptococcus podzolicus	CBS6819[a]	Podzolic soil, type strain of *Cryptococcus podzolicus* (=*Candida podzolica*), Russia	CBS	EF211245	EF211453	EF211573
Tsuchiyaea wingfieldii	CBS7118[a]	Frass of scolytid beetles in *Olea europea* subsp. *africana*, type strain of *Tsuchiyaea wingfieldii* (=*Sterigmatomyces wingfieldii*), South Africa	CBS	EF211246	EF211454	EF211574
Cryptococcus podzolicus	CBS7717	Peat, Russia	CBS	EF211247	EF211455	EF211575
Filobasidiella depauperata	CBS7841[a]	Dead spider, type strain of *Filobasidiella depauperata* (=*Filobasidiella arachnophila*), Canada	CBS	EF211248	EF211456	EF211576

GenBank accession numbers of ITS, *RPB1* and *RPB2* sequences are indicated.
[a] Type strain.

Table 4
Primer sequences, buffer composition and conditions used to determine the mating-type and serotype of *C. neoformans* and *C. gattii* isolates

Specificity	Primer sequences (5′–3′)	Reference	Buffer composition (1× PCR buffer)	Amount of added primer (both primers)	Amplification conditions
Serotype A	JOHE2596:GCCAGAGAGATTCGATGTTG JOHE3241:CATCGCTCCACATCTTCGTT	Lengeler et al. (2001)	10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3	1.0 μM	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, with a final extension step of 72 °C for 5 min
Serotype D	JOHE2596:GCCAGAGAGATTCGATGTTG JOHE3240:TCCACCCCATTCATACCCG	Lengeler et al. (2001)	10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3	1.0 μM	94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 3 min, with a final extension step of 72 °C for 5 min
*MAT*a-serotype A	JOHE7270:ATCAGAGACAGAGGAGGAGCAAGAC JOHE7272:TCCACTGGCAACCCTGCGAG	Barreto de Oliveira et al. (2004)	10.4 mM Tris–HCl, 25 mM KCl, 3.5 mM $MgCl_2$, 5 nM EDTA, pH 9.2	0.5 μM	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
*MAT*α-serotype A	JOHE7264:AGCTGATGCTGTGGATTGAATAC JOHE7265:GTTCAATTAATCTCACTACCTGTAG	Barreto de Oliveira et al. (2004)	10.4 mM Tris–HCl, 25 mM KCl, 3.5 mM $MgCl_2$, 5 nM EDTA, pH 9.2	0.5 μM	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
*MAT*a-serotype D	JOHE7273:GTTCATCAGATACAGAGGAGTGG JOHE7275:CTCCACTGTCAAACCTACGGC	Barreto de Oliveira et al. (2004)	10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3	1.0 μM	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
*MAT*α-serotype D	JOHE7267:ATAGGCTGGTGCTGTGAATTAAG JOHE7268:GTTCAAGTAATCTCACTACATGCG	Barreto de Oliveira et al. (2004)	10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3	1.0 μM	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
*MAT*α-*C. gattii*	MFαU:TTCACTGCCATCTTCACCACC MFαL:TCTAGGCGATGACACAAAGGG	Halliday et al. (1999)	10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3	1.8 μM	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
*MAT*α-*C. gattii*	STE12αU:CAATCTCAAAGCGGGGACAG STE12αL:CTTTGTTTCGGTCCTAATACAGCC	Halliday et al. (1999)	10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3	1.25 μM	94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min, and a final extension step of 72 °C for 7 min

located on chromosome 5, the second largest subunit of RNA polymerase II (*RPB2*) located on chromosome 4, and Translation Elongation Factor 1α (*TEF1*) located on chromosome 13. In addition, the laccase gene (*CNLAC1*) located on chromosome 7 was included in our study. Laccase is an important virulence factor of *C. neoformans* and *C. gattii* (Zhu and Williamson, 2004) and has previously been used to study the molecular systematics of *C. neoformans* and *C. gattii* (Xu et al., 2000).

2.5. Cultivation, DNA extraction, PCR and sequencing

Cultivation and DNA extraction of *C. neoformans* and *C. gattii* isolates was carried out using an optimized protocol of Bolano et al. (2001), which has previously been described (Bovers et al., 2006). *C. amylolentus*, *C. podzolicus*, *F. depauperata* and *T. wingfieldii* isolates were grown on solid YPGA medium (1% yeast extract, 1% pepton, 2% D-glucose, 2% technical agar no. 3; Oxoid, Hampshire, UK) for seven days at room temperature. Cells were harvested and DNA was extracted by adding 750 μl phenol:chloroform:isoamylalcohol (25:24:1, pH 8.0), 750 μl lysis buffer (0.5% w/v SDS, 0.5% w/v Sarkosyl in TE, pH 7.5) and sterile sand to the cells. The cells were bead-beated for 3 min at 2500 beats min^{-1} and subsequently centrifuged for 15 min at 17,000*g* and 4 °C. The DNA fraction was ethanol-precipitated and dissolved in TE buffer.

All PCR amplifications were carried out in a total volume of 50 μl containing 0.2 mM dNTPs, 0.6 μM of both primers, 1.0 U *Taq* DNA polymerase (Gentaur, Brussels,

Table 5
Overview of selected loci and their chromosomal location

Region & chromosomal location	Primer sequences (5′–3′)	Reference	Buffer composition (1× PCR buffer)	Amplification conditions
Internal transcribed spacer 1&2 including 5.8S rDNA (ITS) chromosome 2	ITS1: TCCGTAGGTGAACCTGCGG ITS4: TCCTCCGCTTATTGATATGC	White et al. (1990)	10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3	94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 1 min and 72 °C for 2 min, with a final extension step of 72 °C for 7 min
Intergenic spacer (IGS) chromosome 2	IG1F: CAGACGACTTGAATGGGAACG IG2R: ATGCATAGAAAGCTGTTGG	Diaz et al. (2000)	10.4 mM Tris–HCl, 75 mM KCl, 1.5 mM $MgCl_2$, 5 nM EDTA, pH 8.8	94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 3 min, with a final extension step of 72 °C for 5 min
Laccase (*CNLAC1*) chromosome 7	LAC-F: GGCGATACTATTATCGTA LAC-R: TTCTGGAGTGGCTAGAGC	Xu et al. (2000)	10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3	94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension step of 72 °C for 7 min
Largest subunit of RNA polymerase II (*RPB1*) chromosome 5	RPB1-Af: GARTGYCCDGGDCAYTTYGG RPB1-Cf: CCNGCDATNTCRTTRTCCATRTA	Liu et al. (2006)	10.4 mM Tris–HCl, 75 mM KCl, 3.5 mM $MgCl_2$, 5 nM EDTA, pH 8.3	96 °C for 5 min, followed by 35 cycles of 96 °C for 30 s, 59 °C for 30 s and 72 °C for 2 min, with a final extension step of 72 °C for 5 min
Second largest subunit of RNA polymerase II (*RPB2*) chromosome 4	RPB2-Fcrypto: TGGGGYATGGTTTGTCCKGC RPB2-Rcrypto: CCCATGGCTTGTTTRCCCATYGC	This study	10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3	94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 2 min, with a final extension step of 72 °C for 7 min
Translation elongation factor 1α (*TEF1*) chromosome 13	TEF1-F: AATCGTCAAGGAGACCAACG TEF1-R: CGTCACCAGACTTGACGAAC	Litvintseva et al. (2006)	10.4 mM Tris–HCl, 75 mM KCl, 3.5 mM $MgCl_2$, 5 nM EDTA, pH 8.8	94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min and 72 °C for 2 min, with a final extension step of 72 °C for 7 min

In addition, primer sequences, buffer composition and the conditions used for amplification are shown.

Belgium) and 1–2 μl template DNA. Primer sequences, buffer composition and amplification conditions are listed in Table 5. Amplicons were purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NY) and used for sequencing. Sequencing reactions were carried out with the BigDye v3.1 Chemistry kit (Applied Biosystems, Foster City, CA) using primers that had been used in the initial PCRs. Sequencing reactions were purified with Sephadex G-50 Superfine columns (Amersham Biosciences) and a MultiScreen HV plate (Millipore, Billerica, MA). An ABI 3700XL DNA analyzer (Applied Biosystems) was used to determine the sequences. GenBank accession numbers are listed in Tables 2 and 3.

2.6. Alignment and phylogenetic analyses

SeqMan 5.03 (DNASTAR, Madison, WI) was used to assemble consensus sequences that were checked manually. Sequences were aligned with ClustalX (Thompson et al., 1997) and visually corrected using GeneDoc (www.nrbsc.org/downloads/).

Cryptococcus amylolentus, *C. podzolicus*, *F. depauperata* and *T. wingfieldii* were used as outgroup in ITS, *RPB1* and *RPB2* analyses. *C. neoformans* analyses of IGS1, *CNLAC1* and *TEF1* were carried out using five *C. gattii* isolates (CBS6998, E566, CBS6955, CBS7750, WM779) as outgroups. *C. gattii* analyses of IGS1, *CNLAC1* and *TEF1* were performed using five *C. neoformans* isolates (H99, 125.91, WM714, JEC20, CBS6886) as outgroups. Neighbor-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian (B) phylogenetic analyses were used to analyze ITS, *RPB1* and *RPB2*. IGS1, *CNLAC*1 and *TEF1* were analyzed with NJ and MP phylogenetic analyses.

The optimal substitution model was chosen for every single locus as well as for the concatenated loci using the online software package GARD (Kosakovsky Pond et al., 2006) which tests the models HKY85, TrN93, REV, F81 and GTR. As a result HKY85 fitted the substitution rates in our samples best. Subsequently, NJ and MP phylogenetic analyses were performed using PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b10 software (Swofford, 2000). NJ analyses were carried out using HKY85, as well as the uncorrected ("*p*"), Jukes-Cantor and Kimura 2-parameter substitution models as implemented in PAUP*. Any ties that were encountered were broken randomly. The outcome of the HKY85 substitution model is described in the results section, but support values obtained after the application of the other substitution models implemented in PAUP* are presented in Supplementary Table 1. Bootstrap analysis (Hillis and Bull, 1993) with a 1000 replicates was used to determine the significance of branches. MP analyses were carried out (heuristic search, stepwise addition, random taxon addition, 1000 maximum trees) with tree bisection and reconstruction (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight, and gaps were treated both as missing and as a new character state. The outcome of analyses that treated gaps as a new character state are described in the results section, but all results are presented in the Supplementary Table 1. Gaps in *C. gattii TEF1* and *C. neoformans* IGS1 analyses were treated only as a new character state because of lack of computational power. Bootstrap analysis (Hillis and Bull, 1993) was performed with a 1000 replicates.

ML phylogenetic analyses were carried out using PHYML online (Guindon et al., 2005). Starting trees were generated by BioNJ distance analyses. The outcome of the HKY85 substitution model is described in the results section, but results from other substitution models are presented in the Supplementary Table 1. Bootstrap values were obtained by analyzing 500 bootstrapped pseudo data sets.

Bayesian phylogenetic analyses were performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The transition and transversion substitution rates differed. Each Bayesian analysis was carried out using the default priors and consisted of six runs of 2,000,000 generations and a sample frequency of 100. The trees collected before the stationary phase of the chain was reached were discarded. A consensus tree was generated by combining all remaining trees from the six runs and the posterior probabilities were calculated for each of the clades. A bootstrap value is obtained by multiplying the obtained posterior probability with 100.

2.7. Combinability assessment and concatenation of sequences

One strain was selected from each genotypic group and the distance matrix of each of the six sequenced loci was calculated using ClustalX (Thompson et al., 1997). Pearson's correlation between all of the distance matrices was calculated. Loci with correlation values higher than 0.90 (IGS1, *RPB1*, *RPB2*, *CNLAC1* and *TEF1*) were concatenated and loci with correlation values higher than 0.60 (ITS compared with IGS1, *RPB1*, *RPB2*, *CNLAC1* and *TEF1*) were concatenated as well. A set of concatenated sequences included all haplotypes of either *C. neoformans* or *C. gattii*. In addition, outgroup sequences were included. Four *C. gattii* isolates (WM276, CBS6955, A1M-R265, WM779) representing all haploid genotypic *C. gattii* groups were included as outgroup for the *C. neoformans* data set, whereas two *C. neoformans* isolates (H99, JEC21) representing the two *C. neoformans* varieties were included as outgroup for the *C. gattii* data set. NJ analysis using PAUP* version 4.0b10 (Swofford, 2000) was carried out to compare the results of the sets of concatenated sequences with and without the ITS region. The analyses yielded phylogenetic trees with similar topologies and bootstrap values. We therefore decided to include ITS in all further analyses. NJ, MP, ML and B analyses were carried out as described above.

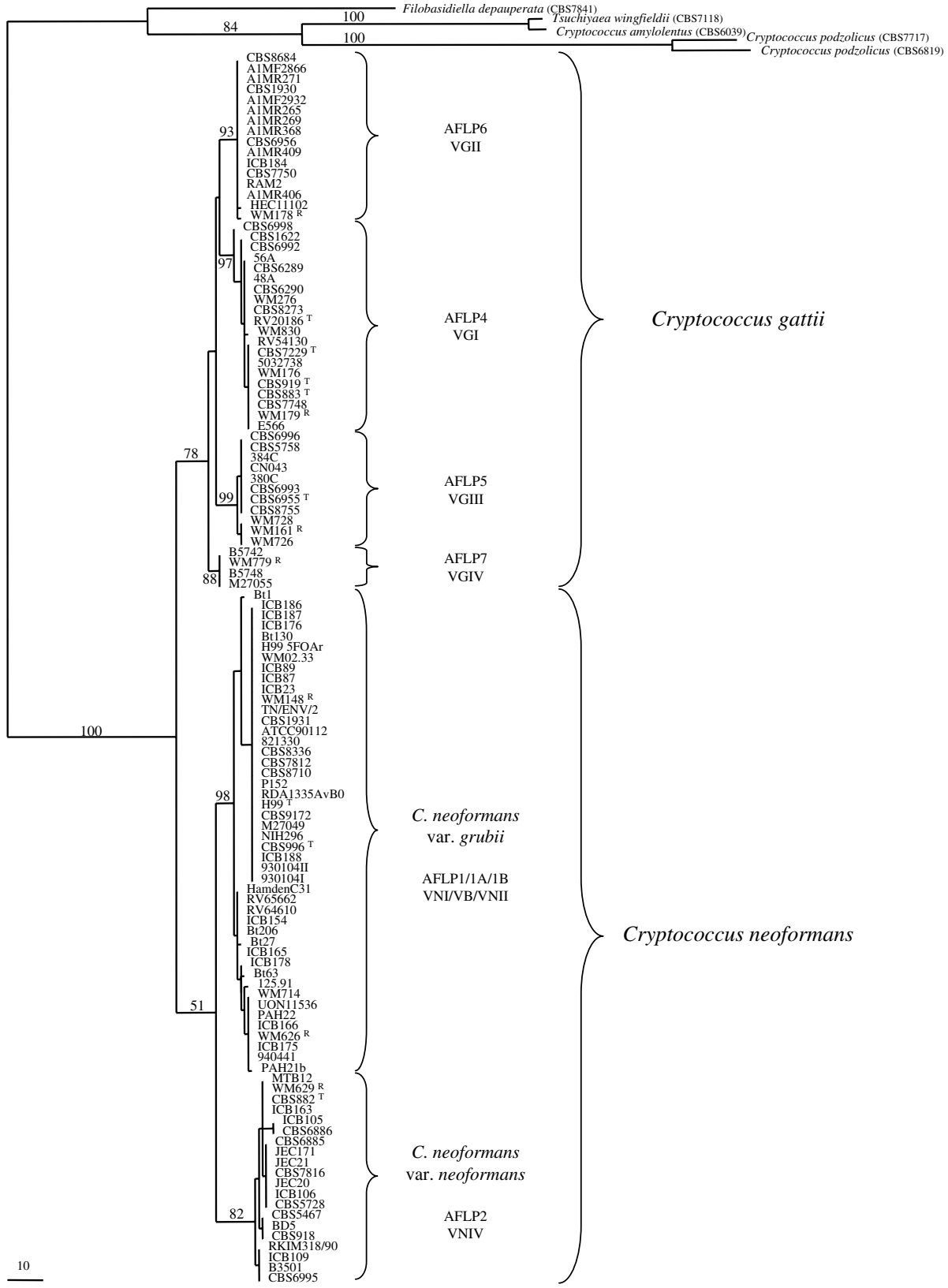

Fig. 1. Phylogenetic tree of *C. neoformans* and *C. gattii* obtained by analysis of partial *RPB2* sequences. Presented is one of 684 most parsimonious trees (length 527; consistency index 0.767; retention index 0.948) computed with gaps treated as new state. Data consisted of 653 characters of which 252 characters were parsimony informative. Bootstrap values (1000 replicates) are indicated for the main branches. ^{T} type strain, ^{R} molecular type reference strain (Meyer et al., 2003).

3. Results

3.1. Ploidy analysis

The ploidy of 43 isolates representing all of the six haploid genotypic groups currently recognized within the *C. neoformans–C. gattii* species complex was determined. The results indicated that eight *C. neoformans* var. *neoformans* isolates were haploid and one *C. neoformans* var. *neoformans* isolate was diploid/aneuploid. Seven *C. neoformans* var. *grubii* isolates were haploid, whereas five *C. neoformans* var. *grubii* isolates were diploid/aneuploid. All 22 *C. gattii* isolates were haploid. The ploidy of the isolates is indicated in Table 2.

3.2. Mating-type and serotype analysis

The mating-type of all one hundred and seventeen isolates included in the study was determined by PCR amplification with mating-type specific primers. Sixteen *C. neoformans* var. *neoformans* isolates belonged to *MAT*α, whereas four *C. neoformans* var. *neoformans* isolates belonged to *MAT*a. Forty-one *MAT*α and five *MAT*a *C. neoformans* var. *grubii* isolates were present. Thirty-nine *C. gattii* isolates belonged to *MAT*α, whereas twelve *C. gattii* isolates belonged to *MAT*a. The mating-type of the isolates is indicated in Table 2.

3.3. Overall genotypic structure of Cryptococcus neoformans and Cryptococcus gattii

The topology of the *C. neoformans–C. gattii* species complex was inferred for ribosomal DNA region ITS and the *RPB1* and *RPB2* genes using NJ, MP, ML and B phylogenetic analyses. Analyses of ITS data resulted in lower bootstrap support than analyses of *RPB1* or *RPB2* data. The topology of the *C. neoformans–C. gattii* species complex obtained by *RPB2* and *RPB1* MP analyses is depicted in Fig. 1 and Supplementary Fig. 1, respectively. An overview of bootstrap values of all analyses performed is given in Supplementary Table 1. The *C. neoformans* cluster received bootstrap support values of 69% for MP, 90% for NJ, 93% for ML and 100% for B analyses. The *C. gattii* cluster was supported by bootstrap values of 93% for ML, 98% for MP and 100% for NJ and B analyses. These results indicate that *C. neoformans* and *C. gattii* represent different groups which appear as sister groups in our phylogenetic analyses. The DNA similarity between *C. neoformans* and *C. gattii* isolates was determined for the combined data set of ITS, IGS1, *CNLAC1*, *RPB1*, *RPB2* and *TEF1*.

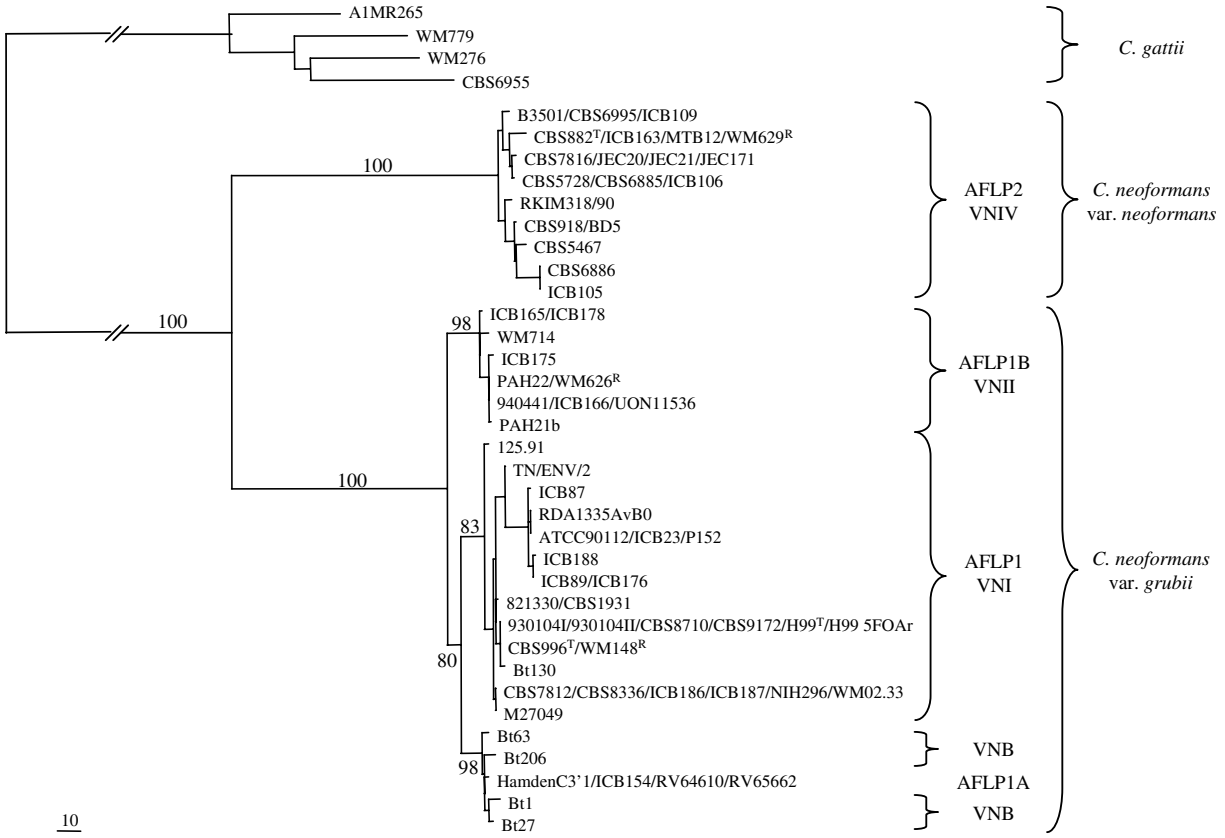

Fig. 2. Phylogenetic tree of both varieties of *C. neoformans* obtained by analysis of the concatenated data set (*RPB1*, *RPB2*, *CNLAC1*, *TEF1*, IGS1 and ITS). Presented is one of 341 most parsimonious trees (length 896; consistency index 0.867; retention index 0.963) computed with gaps treated as new state. Data consisted of 3922 characters of which 550 characters were parsimony informative. Bootstrap values (1000 replicates) are indicated for the main branches. ^{T} type strain, ^{R} molecular type reference strain (Meyer et al., 2003).

C. neoformans and *C. gattii* sequences were found to be 84–86% similar.

3.4. Cryptococcus neoformans genotypic structure

The structure within the *C. neoformans* clade was studied for ribosomal DNA region ITS and the *RPB1* and *RPB2* genes using NJ, MP, ML and B phylogenetic analyses. In addition, NJ and MP phylogenetic analyses were carried out on ribosomal DNA region IGS1, and the *CNLAC1* and *TEF1* genes. The number of *C. neoformans* haplotypes was 4 for ITS, 11 for *CNLAC1*, 12 for *RPB1*, 13 for *RPB2*, 16 for IGS1 and 18 for *TEF1*. All isolates ($N = 66$) clustered consistently, i.e., they fell into either var. *grubii* or var. *neoformans* for all six studied loci. Only the results from *RPB1* analyses are described, but an overview of bootstrap values of all analyses is given in Supplementary Table 1. A separate var. *grubii* cluster was strongly supported by bootstrap values of 99% for MP and 100% for NJ, ML and B analyses. A separate var. *neoformans* branch received bootstrap support of 100% for NJ, MP, ML and B analyses. These results indicate that var. *grubii* and var. *neoformans* represent genetically different groups.

To further infer the relationship of the isolates within the two varieties NJ, MP, ML and B phylogenetic analyses were carried out using the concatenated data set of all six loci. This set included all nine haplotypes of var. *neoformans* ($N = 20$) and all 22 haplotypes of var. *grubii* ($N = 46$). The topology obtained by MP analysis of the concatenated sequences is shown in Fig. 2 and an overview of the obtained bootstrap support values is given in Supplementary Table 2.

The concatenated sequences of the two varieties were 91% to 92% similar. The var. *neoformans* clade contained all AFLP2/VNIV isolates, including the type strain of *Torula nasalis* (CBS882) and the VNIV reference strain (WM629). *T. nasalis* is no longer recognized as a separate species, but is currently considered a synonym of *C. neoformans* var. *neoformans* (Bovers et al., 2008). Two non-supported clusters are present within the var. *neoformans* clade. *MAT*α isolates ($N = 16$) are present in both subclusters, whereas *MAT*a isolates ($N = 4$) are present in only one of the subclusters. Isolates from environmental ($N = 3$), veterinary ($N = 3$), clinical HIV-positive ($N = 2$) and clinical HIV-negative origin ($N = 4$) were present within both of the subclusters. Ploidy analysis on a subset of *C. neoformans* var. *neoformans* isolates ($N = 9$) showed that the majority of the isolates was haploid, but a diploid/aneuploid isolate was found as well. This *MAT*α isolate had been isolated from a HIV-positive patient and possessed only one allele for all six loci analyzed.

Within the var. *grubii* clade three major clusters could be distinguished. The cluster that contained all AFLP1/VNI isolates was well supported by bootstrap values of 83% for MP, 91% for ML, 95% for NJ and 100% for B analyses. The type strain of *C. neoformans* var. *grubii* (H99 = CBS8710 = CBS10515), the type strain of *Candida psicrophylicus* (CBS996), and the reference strain of VNI (WM148) clustered within this cluster. *C. psicrophylicus* is no longer recognized as a separate species, but is currently considered a synonym of *C. neoformans* var. *grubii* (Bovers et al., 2008). The cluster that contained the AFLP1B isolates as well as the reference strain of VNII (WM626) was strongly supported by bootstrap support values of 98% for MP and 100% for NJ, ML and B analyses. Interestingly, all AFLP1A isolates fell into the cluster that also contained the VNB isolates (Litvintseva et al., 2006). This cluster was strongly supported by bootstrap values of 98% for MP, 99% for ML and 100% for NJ and B analyses. The AFLP1/VNI cluster formed a sister group to the AFLP1A/VNB cluster, and this combined cluster received bootstrap support of 69% for NJ, 77% for ML, 80% for MP and 100% for B analyses. *MAT*α isolates ($N = 41$) were present in all three clusters and *MAT*a isolates ($N = 5$) were found in the AFLP1/VNI and the AFLP1A/VNB cluster. Isolates from environmental ($N = 13$), clinical HIV-positive ($N = 15$) and clinical HIV-negative ($N = 10$) origin were present in all three clusters. The only isolate from veterinary origin clustered within the AFLP1B/VNII cluster. Ploidy analysis of a subset of isolates ($N = 12$) showed that four haploid isolates were present in the AFLP1/VNI and three haploid isolates were present in the AFLP1A/VNB cluster. Furthermore, in both the AFLP1/VNI and in the AFLP1A/VNB cluster one diploid/aneuploid isolate was detected. The diploid/aneuploid isolate present in the AFLP1/VNI cluster belonged to *MAT*α and had been isolated from a HIV-positive patient. The diploid/aneuploid isolate present in the AFLP1A/VNB cluster belonged to *MAT*α as well, but had been isolated from a HIV-negative patient. The ploidy of one isolate in the AFLP1B/VNII cluster was determined. This isolate was diploid/aneuploid, belonged to *MAT*α and had been isolated from a cat. All these diploid/aneuploid isolates possessed one allele for each of the six loci analyzed. In addition to these isolates, two diploid/aneuploid isolates were identified that clustered within the AFLP1B/VNII group. In contrast to the other diploid/aneuploid isolates, these isolates possessed two alleles for four of the six loci analyzed. The sequences of these two isolates were identical. Sequence comparison indicated that the sequence of one of the alleles was similar to those of AFLP1B/VNII isolates, whereas the sequence of the other allele was similar to those of AFLP1A/VNB isolates. Both isolates belonged to *MAT*α and had been isolated from pigeon droppings, but they had been isolated from two different Brazilian cities, i.e., São Paulo and Rio de Janeiro.

3.5. Cryptococcus gattii genotypic structure

The structure within the *C. gattii* clade was inferred for ribosomal DNA region ITS and for the *RPB1* and *RPB2* genes by NJ, MP, ML and B analyses. In addition, the structure within the *C. gattii* clade was studied by NJ and MP analyses for ribosomal DNA region IGS1 and the

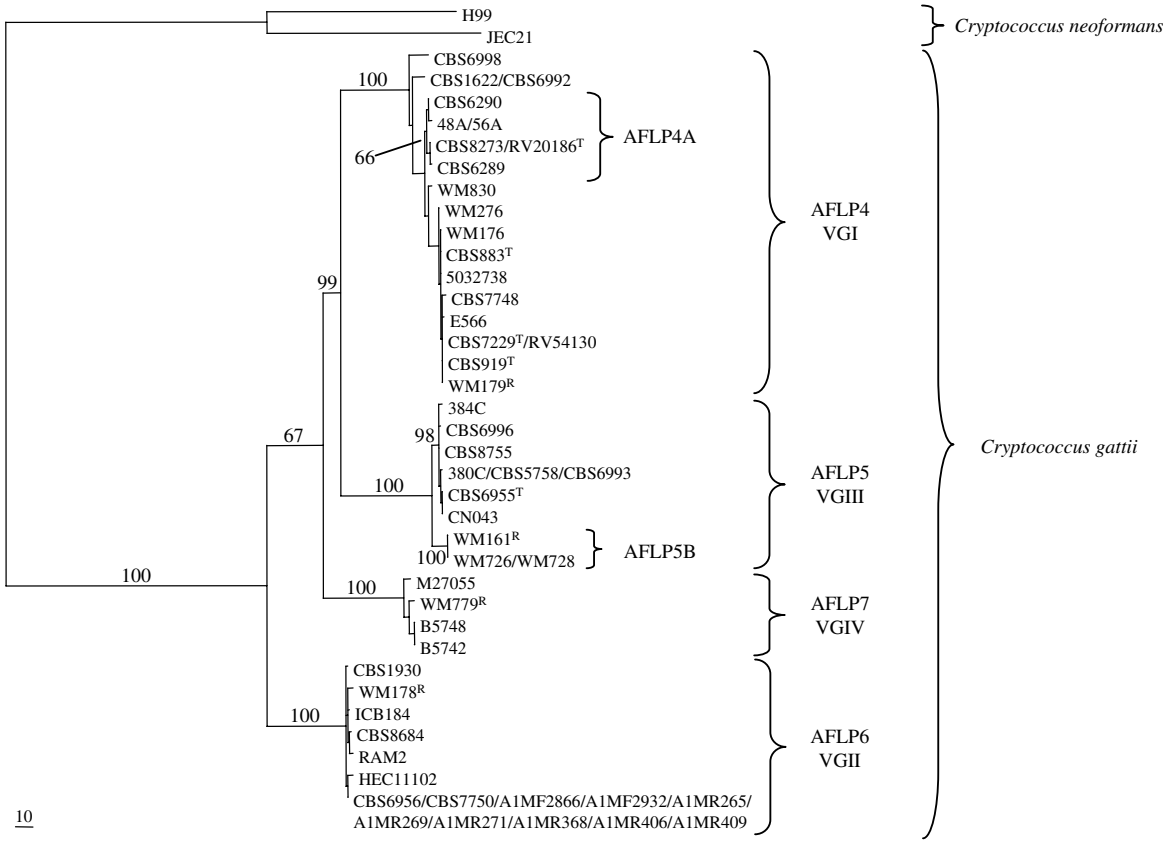

Fig. 3. Phylogenetic tree of *C. gattii* genotypes obtained by analysis of the concatenated data set (*RPB1*, *RPB2*, *CNLAC1*, *TEF1*, IGS1 and ITS). Presented is one of 60 most parsimonious trees (length 847; consistency index 0.902; retention index 0.961) computed with gaps treated as new state. Data consisted of 3932 characters of which 459 characters were parsimony informative. Bootstrap values (1000 replicates) are indicated for the main branches. [T] type strain, [R] molecular type reference strain (Meyer et al., 2003).

CNLAC1 and *TEF1* genes. The number of *C. gattii* haplotypes ($N = 51$) was 9 for ITS, 11 for *RPB1*, 11 for *RPB2*, 16 for *CNLAC1*, 16 for *TEF1* and 20 for IGS1. Ploidy analysis of a subset of isolates ($N = 22$) showed that all investigated *C. gattii* isolates were haploid. Four monophyletic lineages corresponding to the four previously described genotypic groups of *C. gattii* (Meyer et al., 2003) were present in all six loci studied.

All isolates clustered consistently, i.e., they fell into the same monophyletic lineage for all studied loci. Only the results from *RPB1* analyses are described, but an overview of bootstrap values of all analyses is given in Supplementary Table 1. A separate AFLP4/VGI cluster received bootstrap support values of 59% for MP, 64% for ML, 77% for NJ and 93% for B analyses. In addition, a separate AFLP5/VGIII cluster was strongly supported by bootstrap values of 99% for NJ, MP and ML analyses and 100% bootstrap support for B analyses. A separate AFLP6/VGII cluster received high bootstrap values of 98% for ML, 99% for MP and 100% for NJ and B analyses. Furthermore, a separate AFLP7/VGIV cluster received bootstrap support values of 80% for MP, 98% for NJ, 99% for ML and 100% for B analyses. These results indicate that the four genotypic groups of *C. gattii* represent distinct groups.

To infer the relationship of the genotypic groups within the *C. gattii* clade NJ, MP, ML and B phylogenetic analyses were carried out using concatenated sequences of the six loci. All *C. gattii* haplotypes were included, i.e., 13 AFLP4/VGI haplotypes ($N = 20$), 7 AFLP5/VGIII haplotypes ($N = 11$), 7 AFLP6/VGII haplotypes ($N = 16$) and 4 AFLP7/VGIV haplotypes ($N = 4$). The topology of the *C. gattii* clade, derived by MP analysis of the concatenated sequences, is depicted in Fig. 3 and an overview of the bootstrap support values is given in Supplementary Table 3.

The concatenated sequences of the *C. gattii* genotypic groups were 95% to 96% similar. These analyses showed that the AFLP4/VGI and AFLP5/VGIII clusters were sister groups forming a well supported subclade. This subclade received bootstrap support values of 78% for NJ, 88% for ML, 99% for MP and 100% for B analyses. AFLP7/VGIV clustered basal to the AFLP4/VGI and AFLP5/VGIII subclade, and this topology received bootstrap support values of 67% for MP, 89% for NJ and ML analyses and bootstrap support of 100% for B analyses. The AFLP6/VGII cluster clustered basal to the other *C. gattii* genotypic groups.

The previously described AFLP4A and AFLP5B minor genotypes (Boekhout et al., 2001) could be recognized in

our analyses. The type strains of *Torulopsis neoformans* var. *sheppei* (CBS919) and *C. neoformans* var. *shanghaiensis* (CBS7229), as well as the syntype of *Cryptococcus hondurianus* (CBS883), and the reference strain of VGI (WM179) clustered together with the AFLP4 isolates. The above mentioned type strains were once recognized as separate species or varieties, but are currently considered synonyms of *C. gattii* (Bovers et al., 2008). The type strain of *C. gattii* (RV20186 = CBS6289 = CBS8273) clustered in the AFLP4A/VGI subcluster of AFLP4/VGI. Isolates of *MAT*a ($N = 8$) and *MAT*α ($N = 12$) were present within the cluster formed by the AFLP4/VGI isolates and within the subcluster formed by the AFLP4A/VGI isolates. Isolates from clinical origin ($N = 11$) clustered within the AFLP4/VGI cluster as well as within the AFLP4A/VGI subcluster. The AFLP4/VGI isolates ($N = 2$) that had been isolated from immunocompetent patients clustered within the AFLP4/VGI cluster, but not within the AFLP4A/VGI subcluster. The patient's immune status was not known for the other isolates. No environmental AFLP4/VGI isolates ($N = 4$) were present in the AFLP4A/VGI subcluster, but all veterinary isolates ($N = 2$) clustered within the AFLP4A/VGI subcluster.

The type strain of *Cryptococcus bacillisporus* (CBS6955), a current synonym of *C. gattii* (Bovers et al., 2008), clustered together with the AFLP5 isolates. The reference strain of VGIII (WM161) fell into the AFLP5B subcluster of AFLP5. Isolates of *MAT*a ($N = 1$) and *MAT*α ($N = 10$) were present in the cluster formed by AFLP5/VGIII isolates, but the AFLP5B/VGIII subcluster contained only isolates that belonged to *MAT*α. Isolates from clinical origin ($N = 5$) clustered within the AFLP5/VGIII cluster, but no clinical isolates were present within the AFLP5B/VGIII subcluster. The immune status of the patients from which the clinical isolates had been derived is unknown. Isolates from environmental origin ($N = 4$) were present in the AFLP5/VGIII cluster and in the AFLP5B/VGIII subcluster. Isolates from veterinary origin were not present within the AFLP5/VGIII or within the AFLP5B/VGIII subcluster.

The reference strain of VGII (WM178) clustered together with the AFLP6 isolates. Isolates of *MAT*a ($N = 2$) and *MAT*α ($N = 14$) were present in the cluster formed by AFLP6/VGII isolates. The AFLP6/VGII cluster contained isolates from veterinary ($N = 2$), environmental ($N = 4$) and clinical ($N = 10$) origin. The immune status of the patient of one isolate was not known, all other clinical isolates had been isolated from immunocompetent patients.

The reference strain of VGIV (WM779) clustered together with the AFLP7 isolates. The AFLP7/VGIV cluster contained isolates from veterinary ($N = 1$) and clinical ($N = 3$) origin, and were all *MAT*α ($N = 4$) isolates. Isolates from environmental origin were not present within this cluster. One of the clinical isolates had been obtained from an HIV-infected patient, but the patient's immune status of two other isolates was unknown.

4. Discussion

Our analyses showed that six monophyletic lineages, that correspond to the previously recognized molecular genotypes (Boekhout et al., 2001; Meyer et al., 2003; Diaz et al., 2005), occur consistently within the *C. neoformans–C. gattii* species complex. Sequence similarity of the concatenated data set was 91% to 92% between the two *C. neoformans* varieties and 95% to 96% between the *C. gattii* genotypes. The sequence similarity between *C. neoformans* and *C. gattii* was 84% to 86%. These observed similarities lie between those observed in other studies (Aulakh et al., 1981; Butler and Poulter, 2005; Chaturvedi et al., 2005; Diaz et al., 2005; Kavanaugh et al., 2006). Sequence similarities may differ between loci. A recent study showed that the two varieties of *C. neoformans* share 85% to 90% nucleotide identity at the genomic level (Kavanaugh et al., 2006). Interestingly, this study also identified a 14 gene region that is nearly identical between the two *C. neoformans* varieties. This region probably introgressed from *C. neoformans* var. *grubii* into *C. neoformans* var. *neoformans* through an intermediate AD hybrid. In this AD hybrid the 14 gene region was exchanged and remaining *C. neoformans* var. *grubii* chromosomes were lost, thus generating a *C. neoformans* var. *neoformans* with a 14 gene region from *C. neoformans* var. *grubii* (Kavanaugh et al., 2006).

Previous phylogenetic analyses of several coding and non-coding regions, i.e., ITS, IGS, orotidine monophosphate pyrophosphorylase (*URA5*), mitochondrial cytochrome *b* (*cyt b*), mitochondrial large ribosomal subunit RNA (MtLrRNA), *PRP8* intein, mating-pheromone (*MF*α), topoisomerase (*TOP1*), laccase (*LAC*) and a capsule gene (*CAP59*) demonstrated that *C. gattii, C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* form distinct clusters (Xu et al., 2000; Sugita et al., 2001; Chaturvedi et al., 2002; Biswas et al., 2003; Katsu et al., 2004; Butler and Poulter, 2005; Diaz et al., 2005). Our phylogenetic analyses confirm the existence of distinct clusters for *C. gattii*, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* for all six nuclear loci. In addition, analyses of six nuclear loci as well as analyses of the *C. neoformans* concatenated data set showed that *C. neoformans* var. *grubii* formed a sister group to *C. neoformans* var. *neoformans*. Our data thus confirms the topology of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* that has been observed in studies on ITS, IGS, *cyt b*, MtLrRNA, *PRP8* intein, *TOP1*, and *LAC* (Xu et al., 2000; Sugita et al., 2001; Biswas et al., 2003; Katsu et al., 2004; Butler and Poulter, 2005; Diaz et al., 2005).

Phylogenetic analysis of the *C. neoformans* concatenated data set also revealed the presence of three clusters within the var. *grubii* clade. These clusters correspond to the previously described AFLP genotypes 1, 1A and 1B (Boekhout et al., 2001; Barreto de Oliveira et al., 2004). The reference strain of VNI clustered together with AFLP1. The reference strain of VNII fell into the AFLP1B cluster, which indicates that genotype VNII is identical to

AFLP1B. Genotype VNII was previously thought to correspond to the AFLP1A genotype, but at that time the AFLP1B genotype had not been recognized (Boekhout et al., 2001; Meyer et al., 2003; Barreto de Oliveira et al., 2004). Surprisingly, the AFLP1A isolates in our study ($N = 4$) clustered together with the VNB isolates, which originated from Botswana. The AFLP fingerprints of the VNB isolates (data not shown) are similar to those of the AFLP1A isolates (Boekhout et al., 2001). In addition, comparison of AFLP1A sequences with additional IGS1 and *CNLAC1* VNB sequences (Litvintseva et al., 2006) confirmed that the AFLP1A isolates clustered within the VNB cluster. So far, the VNB genotype had only been found in haploid isolates in Botswana (Litvintseva et al., 2006). In addition, the VNB genotype was detected in serotype A *MAT*a-serotype D *MAT*α hybrids from Italy, China and the USA (Litvintseva et al., 2007). These hybrids probably originated from a intervarietal mating between a *C. neoformans* var. *grubii* VNB isolate and a *C. neoformans* var. *neoformans* isolate. The serotype A *MAT*a-serotype D *MAT*α hybrids possess an increased fitness and it has been speculated that they spread from sub-Saharan Africa to other continents (Litvintseva et al., 2007). The AFLP1A/VNB isolates present in our study had been isolated from Brazilian pigeon droppings and from patients in Rwanda, Portugal/Venezuela and Brazil. However, it is unlikely that these isolates resulted from the above mentioned intervarietal hybridization since all four isolates belonged to *MAT*α. Our results indicate that the occurrence of the AFLP1A/VNB genotype in haploid isolates is not restricted to Africa, but show that they may also occur in South America. All four AFLP1A/VNB isolates belonged to *MAT*α and their sequences were identical for all six loci analyzed. Three subgroups, namely VNB-A, VNB-B and VNB-C, have been identified within the VNB cluster (Litvintseva et al., 2006). Sequence comparison indicated that the four AFLP1A/VNB isolates were more closely related to isolates belonging to the VNB-A subgroup, i.e., Bt1, Bt27 and Bt206, than to isolates belonging to the VNB-B subgroup, i.e., Bt63. Studies carried out by Litvintseva et al. (2006) demonstrated that a high proportion of *MAT*a isolates was present within the VNB genotype. However, the distribution of mating-type was uneven: the VNB-B subgroup was dominated by *MAT*a isolates, whereas the VNB-A subgroup was dominated by *MAT*α isolates (Litvintseva et al., 2006). The four AFLP1A/VNB isolates, which are most closely related to the VNB-A subgroup, all possessed the *MAT*α allele, which fits the previously published results.

Ploidy analysis of a subset of *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* isolates showed that the majority of these isolates, including three of the four AFLP1A/VNB isolates, were haploid. In addition to the haploid isolates, a diploid/aneuploid isolate was identified in the *C. neoformans* var. *neoformans* cluster, and in the *C. neoformans* var. *grubii* AFLP1/VNI, AFLP1B/VNII and AFLP1A/VNB subclusters. All four diploid/aneuploid isolates within the *C. neoformans* cluster belonged to *MAT*α and possessed one allele for each of the six loci analyzed. Other diploid/aneuploid *C. neoformans* isolates, which posses only one mating-type allele, have been described (Cogliati et al., 2001; Ohkusu et al., 2002; Litvintseva et al., 2003). The presence of only one mating-type allele and the identification of only one allele for all six studied loci suggest that these isolates may have originated from auto-diploidization or same-sex mating. Same-sex mating has been suggested as a mechanism that can enhance adaptation to changing environments (Lin et al., 2005). Therefore, the discovery of an environmental diploid *MAT*α *C. gattii* isolate from Vancouver Island that was homozygous for thirty loci led to the suggestion that the cryptococcosis outbreak on Vancouver Island resulted from same-sex mating (Fraser et al., 2005). In addition, the isolation of serotype A *MAT*α-serotype D *MAT*α hybrids from the environment indicates that same-sex mating occurs in nature (Litvintseva et al., 2005a; Lin et al., 2007). Unlike the above-mentioned environmental isolates, the diploid *C. neoformans* isolates present in our study had been isolated from a cat and from both HIV-negative and HIV-positive patients. In addition, two diploid/aneuploid *C. neoformans* var. *grubii* isolates were identified that possessed two alleles for four of the six loci analyzed. These isolates possessed only one mating-type allele and may have resulted from same-sex mating (Lin et al., 2005, 2007). Results obtained by sequence comparison indicated that the parents of these two isolates probably belonged to AFLP1B/VNII and AFLP1A/VNB. These isolates had been isolated from pigeon droppings in São Paulo and Rio de Janeiro. Recently, a MLST study on *C. neoformans* var. *grubii* isolates identified nine isolates that were inconsistently placed among genealogies and possessed alleles corresponding to AFLP1/VNI and AFLP1A/VNB isolates suggesting that mating between the AFLP1/VNI and AFLP1A/VNB may occur in the environment (Litvintseva et al., 2006). The discovery of diploid AFLP1B/VNII-AFLP1A/VNB isolates suggests that mating between AFLP1B/VNII and AFLP1A/VNB may also occur in the environment, thus confirming laboratory experiments which showed that AFLP1A/VNB isolates are capable of mating with AFLP1B/VNII isolates (Litvintseva et al., 2006). Apparently, AFLP1A/VNB isolates are capable of mating with isolates that belong to both of the other two *C. neoformans* var. *grubii* genotypes.

Phylogenetic analysis of the *C. neoformans* concatenated data set also showed that the AFLP1/VNI and AFLP1A/VNB clusters are sister groups and indicated that the AFLP1B/VNII cluster is basal to the other two clusters. Our data thus support the topology that has been found in a MLST study of *C. neoformans* var. *grubii* isolates using twelve unlinked loci (Litvintseva et al., 2006).

The *C. gattii* isolates formed four strongly supported monophyletic lineages for all six nuclear loci studied. Several studies have shown that isolates of both serotype B and C are present in a single *C. gattii* genotype (a.o.

Meyer et al., 2003; Fraser et al., 2005). In our study, isolates of both serotype B and serotype C were present in the AFLP5/VGIII and the AFLP7/VGIV lineage. In contrast to the situation in *C. neoformans*, were serotype and genotype are correlated, these results show that serotype and genotype are not correlated in *C. gattii*. Ploidy analysis carried out on a subset of 22 *C. gattii* isolates that represented all four *C. gattii* genotypes showed that all isolates were haploid. Although a diploid *C. gattii* isolate has been described (Fraser et al., 2005), no diploid/aneuploid *C. gattii* isolates were observed in our study.

All *C. gattii* isolates clustered in the same monophyletic lineage for all loci studied. *C. gattii* has consistently been divided into four genotypes, using molecular fingerprinting methods (Ruma et al., 1996; Ellis et al., 2000; Latouche et al., 2003; Meyer et al., 2003) and sequence analysis (Chaturvedi et al., 2002; Biswas et al., 2003; Diaz et al., 2005; Butler and Poulter, 2005; Fraser et al., 2005; Kidd et al., 2005; this study). Therefore, we propose to recognize these genotypes as different taxa, which will provide a context to better understand the biological and epidemiological characteristics of each of these groups. Some of these characteristics are already known, e.g., AFLP4/VGI is the *C. gattii* parental genotype present in all currently known *C. neoformans–C. gattii* hybrids (Bovers et al., 2006; Bovers et al., 2008) and AFLP6/VGII is the *C. gattii* genotype responsible for the ongoing outbreak of cryptococcosis on Vancouver Island (Kidd et al., 2004). Furthermore, AFLP7/VGIV might be associated with infections in HIV-positive patients as all *C. gattii* isolates that have been isolated from HIV-infected patients in sub-Saharan Africa belonged to *C. gattii* AFLP7/VGIV (Litvintseva et al., 2005b; F. Hagen and T. Boekhout, unpubl. observ.). In addition, the only *C. gattii* isolate in our study that had been isolated from a HIV-positive patient belonged to AFLP7/VGIV. Additional characteristics will be identified more easily when the *C. gattii* genotypes are recognized as separate taxa.

Analyses were carried out to infer the relationship among the *C. gattii* genotypic groups using the concatenated data set. These analyses indicated that AFLP7/VGIV clusters basal to the AFLP4/VGI and AFLP5/VGIII sister groups and that AFLP6/VGII is basal to all other *C. gattii* genotypic groups. The same topology has been found in analyses of the IGS1 + 5S + IGS2 (Diaz et al., 2005) and *TEF1* region (Fraser et al., 2005). In addition, *PRP8* intein and *URA5* analyses supported a topology were AFLP7/VGIV clustered basal to the AFLP4/VGI and AFLP5/VGIII sister groups (Butler and Poulter, 2005; Kidd et al., 2005). Furthermore, AFLP4/VGI and AFLP5/VGIII formed sister groups in *GPD1* analysis (Fraser et al., 2005). However, *C. gattii* topologies that are in conflict with the above mentioned topology have been described as well. *CNLAC1* analysis indicated that AFLP4/VGI clustered basal to the AFLP6/VGII and AFLP7/VGIV sister groups (Fraser et al., 2005; Kidd et al., 2005; this study). In addition, the topology obtained by *FTR* analysis showed that AFLP4/VGI was basal to the AFLP5/VGIII and AFLP7/VGIV sister groups (Kidd et al., 2005). Furthermore, IGS1 analysis showed that AFLP6/VGII clustered basal to the AFLP4/VGI and AFLP5/VGIII sister groups (this study), but analysis of the complete IGS1 + 5S + IGS2 region (Diaz et al., 2005) resulted in a topology identical to the topology obtained with our concatenated data set. Although phylogenetic analyses of different nuclear loci have resulted in several different tree topologies, analysis of concatenated data sets consisting of four loci (Kidd et al., 2005) and consisting of six loci (this study) resulted in the same topology. As these studies have used different loci, we consider the topology that has been obtained using the concatenated data sets as the most accurate topology. This topology indicates that AFLP4/VGI and AFLP5/VGIII are the two *C. gattii* groups that are most closely related, whereas AFLP6/VGII is the most basal group in the *C. gattii* cluster.

Population studies of *C. neoformans* and *C. gattii* indicated that recombination may occur within subpopulations of the same genotype (Litvintseva et al., 2003; Campbell et al., 2005a; Litvintseva et al., 2005a). In addition, the high amount of karyotype variability, which has been observed within *C. neoformans* and *C. gattii* (Kwon-Chung et al., 1992b; Perfect et al., 1993; Dromer et al., 1994; Boekhout et al., 1997), may result from recombination as karyotype polymorphisms are usually generated by meiotic events (Zolan, 1995). Our data showed that six monophyletic lineages were consistently present within the *C. neoformans–C. gattii* species complex. In addition, all isolates clustered in the same monophyletic lineage for all loci studied, thus indicating that recombination between monophyletic lineages has not occurred. Based on these results the monophyletic lineages should be described as separate taxa. Many different species concepts could be used to determine the status of these monophyletic lineages, but the two most widely used are the Genealogical Concordance Phylogenetic Species Concept (GCPSC) and the Biological Species Concept (BSC). The GCPSC defines a species as "a basal, exclusive group of organisms all of whose genes coalesce more recently with each other than with those of any organism outside the group, and that contains no exclusive group within it" (Baum and Donoghue, 1995; Taylor et al., 2000). Organisms that fall into one monophyletic lineage for all genes studied are considered different species under the GCPSC. The BSC defines a species as "a group of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Mayr, 1940; Taylor et al., 2000). Under the BSC organisms that may sexually reproduce and produce fertile progeny are considered a species. Our data showed that all isolates clustered consistently in the same monophyletic lineage for all loci studied, thus indicating absence of recombination between different monophyletic lineages. According to the GCPSC the monophyletic lineages should thus be described as different species. However, when the BSC is applied, the description of each of the six monophyletic

lineages as separate species can be discussed. The existence of hybrids between the two varieties of *C. neoformans* (Tanaka et al., 1999; Lengeler et al., 2001), and the recent discovery of hybrids between *C. neoformans* and *C. gattii* (Bovers et al., 2006; Bovers et al., in press) indicates that mating, including conjugation and nuclear fusion, occurs between the two varieties of *C. neoformans* and between *C. neoformans* and *C. gattii* in the environment. Surprisingly, so far recombinants of these combinations have not been observed to occur in nature. However, laboratory crossings between *C. neoformans* var. *neoformans* and *C. gattii* AFLP5/VGIII or *C. gattii* AFLP6/VGII resulted in the formation of basidiospores, but these were not studied in more detail (Schmeding et al., 1981; Kwon-Chung et al., 1982; Fraser et al., 2003; Campbell et al., 2005b). The progeny resulting from a mating between *C. neoformans* var. *neoformans* (B3502) and *C. gattii* AFLP4/VGI (CBS6289) was studied more closely (Kwon-Chung and Varma, 2006). No recombinant haploid progeny was found among the sixteen isolates studied (Kwon-Chung and Varma, 2006) confirming that *C. neoformans* and *C. gattii* are different species. The progeny resulting from mating between *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* was studied as well (Cogliati et al., 2006; Kwon-Chung and Varma, 2006; Sun and Xu, 2007). Haploid recombinant isolates were not recovered among ten studied isolates (Cogliati et al., 2006), but three isolates possessed a recombinant genotype among twenty isolates studied (Kwon-Chung and Varma, 2006). Sun and Xu (2007) investigated 115 loci for 163 isolates resulting from a mating between *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*. Hundred and sixty-two isolates possessed at least one heterozygous locus indicating that these isolates are diploid or aneuploid. The high amount of hybrids and the low recombination frequency found in this mating suggest that abnormal or incomplete nuclear disjunctions occurred during meiosis (Sun and Xu, 2007). In addition, comparison of the genomes of *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* shows that there has not been any recent DNA exchange between these two varieties (Kavanaugh et al., 2006). Altogether, these results indicate that the two varieties of *C. neoformans* have diverged to such an extent that normal mating is no longer possible thus indicating that they should be considered different species. As the naming of these two species is complex, because the type strain of *C. neoformans* CBS132 is an AD hybrid, this will be elaborated elsewhere.

The description of the teleomorph of *C. gattii*, i.e. *Filobasidiella bacillispora*, was based on the observation of basidiospores resulting from mating between *C. gattii* AFLP5/VGIII (CBS6955) and *C. gattii* AFLP6/VGII (CBS6956) (Kwon-Chung, 1976b; Boekhout et al., 2001). Furthermore, basidiospores were observed in several crosses between *C. gattii* AFLP5/VGIII and *C. gattii* AFLP6/VGII isolates (Schmeding et al., 1981; Fraser et al., 2003; Campbell et al., 2005b) and in crosses between *C. gattii* AFLP4/VGI and *C. gattii* AFLP5/VGIII (Kwon-Chung et al., 1982; Fraser et al., 2003). Unfortunately, these studies did not investigate the progeny in more detail. Although the above described studies indicate that mating may occur between different genotypic groups of *C. gattii*, no data is present on the fertility of the obtained progeny. To investigate whether the genotypic groups are different species, studies should be carried out to investigate the fertility and genotype of the progeny in more detail. In addition, studies should be carried out on locations were different genotypes are present to investigate whether mating between different *C. gattii* genotypic groups may occur in nature. Although it is clear that the *C. gattii* genotypic groups represent different taxa, more information is needed to determine whether the genotypic groups of *C. gattii* are different species according to the BSC. In conclusion, our data showed that six monophyletic lineages were consistently present within the *C. neoformans–C. gattii* species complex indicating that these lineages should be described as separate taxa. Our results combined with results from other studies indicate that the two varieties of *C. neoformans* should be described as different species. However, to determine the taxonomic status of the various monophyletic lineages present in *C. gattii*, further information is needed on the fertility and genotype of progeny resulting from intergenotypic matings within *C. gattii*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2007.12.004.

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