RESEARCH ARTICLE

Promiscuous mitochondria in *Cryptococcus gattii*

Marjan Bovers[1], Ferry Hagen[1,2], Eiko E. Kuramae[1] & Teun Boekhout[1,2]

[1]CBS Fungal Biodiversity Centre, Uppsalalaan, Utrecht, The Netherlands; and [2]Division of Acute Medicine and Infectious Diseases, Eijkman-Winkler Centre for Medical Microbiology, Infectious Diseases and Inflammation, University Medical Centre, Utrecht, The Netherlands

Correspondence: Teun Boekhout, CBS Fungal Diversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. Tel.: +31 30 212 671; fax: +31 30 251 2097, e-mail: t.boekhout@cbs.knaw.nl

Present address: Eiko E. Kuramae, Netherlands Institute of Ecology, Centre for Terrestrial Ecology, 6666 ZG Heteren, The Netherlands.

Received 3 October 2008; revised 27 January 2009; accepted 30 January 2009.
First published online 5 March 2009.

DOI:10.1111/j.1567-1364.2009.00494.x

Editor: Cletus Kurtzman

Keywords
Cryptococcus gattii; pathogen; mitochondria; recombination.

Abstract

Cryptococcus gattii is a primary pathogenic basidiomycetous yeast comprising four genotypic groups. Here we present data on two mitochondrial loci (MtLrRNA and *ATP6*). Two of the genotypic groups, namely amplified fragment length polymorphism (AFLP)5/VGIII and AFLP6/VGII, formed monophyletic lineages. The AFLP4/VGI genotypic group, however, possessed five different mitochondrial genotypes that did not form a monophyletic lineage. The majority of these isolates contained mitochondrial genomes that are partially identical to those found in isolates belonging to AFLP6/VGII, which is causing the ongoing and expanding Vancouver Island outbreak. Two out of four AFLP7/VGIV isolates contained an AFLP4/VGI allele of MtLrRNA. These observations are best explained by assuming a process of mitochondrial recombination. If this is true, mitochondrial recombination seems possible between cells belonging to different genotypic groups of *C. gattii*, especially between AFLP6/VGII or AFLP7/VGIV and AFLP4/VGI. We also have to assume that mitochondria, most likely, were transferred from cells belonging to AFLP6/VGII to AFLP4/VGI. As such a process of mitochondrial recombination is only possible after cell–cell conjugation, this may also allow the further exchange of genetic material, for example nuclear or plasmid in nature, between different genotypes of *C. gattii*. This may be relevant as it may provide a possible mechanism contributing to the modulation of virulence attributes of isolates, such as has been observed in the ongoing Vancouver Island outbreak of *C. gattii*.

Introduction

Cryptococcus gattii is a primary pathogenic basidiomycetous yeast that has previously been considered a variety of *Cryptococcus neoformans* (Kwon-Chung *et al.*, 1982). Recently, *C. gattii* has been described as a distinct species because of differences in ecology, epidemiology, biochemical and molecular characteristics (Kwon-Chung *et al.*, 2002; Kwon-Chung & Varma, 2006). Both *C. gattii* and *C. neoformans* may cause meningoencephalitis, but the primary pathogen *C. gattii* can cause disease in otherwise healthy individuals, whereas the opportunist *C. neoformans* primarily infects immunocompromised patients (Speed & Dunt, 1995). *Cryptococcus gattii* has been found on several tree species since its first isolation from *Eucalyptus camaldulensis* (Ellis & Pfeiffer, 1990; Callejas *et al.*, 1998; Lazéra *et al.*, 1998, 2000; Fortes *et al.*, 2001; Krockenberger *et al.*, 2002; Fraser *et al.*, 2003; Randhawa *et al.*, 2003; Granados & Castañeda, 2005; Escandón *et al.*, 2006; Kidd *et al.*, 2007), suggesting that trees might be the primary ecological niche of the species. In contrast, Kidd *et al.* (2007) suggested that soil may be the principal reservoir for *C. gattii. Cryptococcus gattii* occurs predominantly in subtropical areas (Kwon-Chung & Bennett, 1984), but has also been isolated in Europe (Viviani *et al.*, 2006) and in a temperate climate zone in Colombia (Escandón *et al.*, 2006). Furthermore, *C. gattii* is responsible for the ongoing outbreak of cryptococcosis on Vancouver Island, Canada (Stephen *et al.*, 2002; Hoang *et al.*, 2004; Kidd *et al.*, 2004) and the further spread of this outbreak to the Pacific Northwest of the United States (MacDougall *et al.*, 2007; Upton *et al.*, 2007). Taken together, these reports indicate that *C. gattii* also occur in more temperate climate zones.

Several molecular methods have identified four major genotypic groups within *C. gattii* (Ruma *et al.*, 1996; Ellis *et al.*, 2000; Chaturvedi *et al.*, 2002; Biswas *et al.*, 2003;

Table 1. Overview of *Cryptococcus gattii* genotypes as identified by several molecular methods

Serotype*	AFLP genotype†,‡,§	Molecular genotype*,¶	IGS genotype‖	ITS genotype**	Intein genotype††
B/(C)‡‡	4A/4B	VGI	4a/4b/4c	3/7	ig-I
B/C	5A/5B/5C	VGIII	5	5	ig-III
B/(C)‡‡	6	VGII	3	4	ig-II
B/C	7	VGIV	6	6	ig-IV

*Meyer *et al*. (2003).
†Barreto de Oliveira *et al*. (2004).
‡Boekhout *et al*. (2001).
§Kidd *et al*. (2004).
¶Litvintseva *et al*. (2006).
‖Diaz *et al*. (2005).
**Katsu *et al*. (2004).
††Butler & Poulter (2005).
‡‡Serotypes indicated between parentheses were not included in our study.

Latouche *et al*., 2003; Meyer *et al*., 2003; Butler & Poulter, 2005; Diaz *et al*., 2005; Fraser *et al*., 2005; Kidd *et al*., 2005; Bovers *et al*., 2008b, c). An overview of these genotypic groups is given in Table 1. All these studies used nuclear regions to study the genotypic structure of *C. gattii*. Mitochondrial regions are useful genetic markers as well, because mitochondria evolve independently from the nuclear genome and thus provide an additional, independent dataset. In *C. neoformans*, mitochondria are usually uniparentally inherited from the *MATa* parent, although mitochondrial inheritance from the *MAT*α parent may also occur at low frequency (Xu *et al*., 2000; Yan & Xu, 2003; Toffaletti *et al*., 2004; Yan *et al*., 2004, 2007a, b). In our study, the partial nucleotide sequence of two mitochondrial regions, namely large ribosomal subunit RNA (MtLrRNA) and ATP synthase subunit 6 (*ATP6*), was investigated for 51 isolates representing all *C. gattii* genotypic groups. The sequences obtained were used for phylogenetic analyses and the resulting genealogies were compared with the genealogy obtained by analyses of six nuclear regions (Bovers *et al*., 2008b). Surprisingly, the analysis of the mitochondrial regions showed that the majority of amplified fragment length polymorphism AFLP4/VGI isolates studied possessed a mitochondrial genome that contained sequences that were partially identical to those found in AFLP6/VGII isolates. These findings are discussed and explained by assuming a process of mitochondrial recombination between the different genotypic groups of *C. gattii*.

Materials and methods

Isolates

Fifty-one haploid isolates belonging to all *C. gattii* genotypic groups from clinical (59%), veterinary (8%), environmental (24%), laboratory (6%) and unknown (3%) origin were analyzed (Table 2). *Cryptococcus neoformans* isolates (125.91, H99, WM714, CBS6886 and JEC20) were used as outgroup in phylogenetic analyses, as *C. neoformans* is the species that is most closely related to *C. gattii* (Fell *et al*., 2000; Kwon-Chung *et al*., 2002).

Cultivation, DNA extraction, PCR reaction 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 been described previously (Bovers *et al*., 2006). PCR reactions were performed in a total volume of 50 μL containing 10 × 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, 0.6 μM of primer, 1.0 U *Taq* DNA polymerase (Gentaur, Brussels, Belgium) and 1–2 μL genomic DNA. Amplification of MtLrRNA was carried out using primers MtLrRNA-F (5′-GACCCTATGCA GCTTCTACTG-3′) and MtLrRNA-R (5′-TTATCCCTAGC GTAACTTTTATC-3′) (White *et al*., 1990). Amplification conditions were 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 5 min. Mitochondrial *ATP6* was amplified using primers ATP6-F (5′-ATTACATCTCC ACTAGAACAATTC-3′) and ATP6-R (5′-AGTTCAATGGC ATCCTTGATATAG-3′). Amplification conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min, with a final extension step of 72 °C for 5 min. Amplicons were purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NY) and used for sequencing. Sequencing reactions were performed with the BigDye v3.1 Chemistry kit (Applied Biosystems, Foster City, CA) using primers that had been used in the initial PCR reactions. Sequencing reactions were purified with Sephadex G-50

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

Isolate	Origin	Sero-type	Mating type	Nuclear genotype*	AFLP geno-type†,‡,§	Mole-cular type¶	MtLrRNA haplotype	*ATP6* haplo-type	References /sources	MtLrRNA	*ATP6*	ITS	IGS	*RPB1*	*RPB2*	*CNLAC1*	*TEF1α*
C. gattii AFLP4 = VGI																	
48A (=CBS11230)	Lung of a goat, Spain	B	α	4A	4A	ND	2	4	Diaz *et al.* (2000)	EF544832	EF544778	EF211195	AJ300934‖	EF211403	EF211523	EF211643	EF211758
503 2738 (=WM1251)	Human, Papua, New Guinea	B	α	4	4	VGI	2	1	Katsu *et al.* (2004)	EF544833	EF544779	EF211196	EF211302	EF211404	EF211524	EF211644	EF211759
56A (=CBS11231)	Gut of a goat, Spain	B	α	4A	4A	ND	2	4	Diaz *et al.* (2000)	EF544834	EF544780	EF211197	AJ300932‖	EF211405	EF211525	EF211645	EF211760
CBS883 (T)	Infected skin, syntype *Cryptococcus hondurianus*, Honduras	B	α	4	4B	ND	4	2	Boekhout *et al.* (1997)	EF544835	EF544781	EF211198	EF211303	EF211406	EF211526	EF211646	EF211761
CBS919 (T)	Meningoencephalic lesion, type strain of *Torulopsis neoformans* var. *sheppei*	B	α	4	4B	ND	4	2	Boekhout *et al.* (1997)	EF544836	EF544782	EF211199	AJ300928‖	EF211407	EF211527	EF211647	EF211762
CBS1622	Man, Tumour, Lille, France	B	α	4	4B	ND	2	3	Boekhout *et al.* (1997)	EF102067**	EF102038**	EF102028**	EF102032**	EF102061**	EF102051**	EF102071**	EF102047**
CBS6289 (=CBS8273, =RV20186, = NIHB-3939)	Subculture of type strain of *Cryptococcus gattii* (RV 20186)	B	**a**	4A	4A	ND	2	4	Boekhout *et al.* (1997)	EF544837	EF544783	EF211200	AJ300937‖	EF211408	EF211528	EF211648	EF211763
CBS6290	Man, Congo (Zaire)	B	α	4A	4A	ND	2	4	Boekhout *et al.* (1997)	EF544838	EF544784	EF211201	AJ300930‖	EF211409	EF211529	EF211649	EF211764
CBS6992 (=NIH 17)	Man	B	α	4	4B	ND	2	3	Boekhout *et al.* (1997)	EF102068**	EF102039**	EF102029**	AJ300923‖/ EF102033**	EF102062**	EF102052**	EF102072**	EF102048**
CBS6998 (=NIH 365)	Human, Thailand	B	**a**	4	4	ND	2	4	Boekhout *et al.* (1997)	EF544839	EF544785	EF211202	AJ300925‖	EF211410	EF211530	EF211650	EF211765
CBS7229 (T)	Meningitis, type strain *Cryptococcus neoformans* var. *shanghaiensis*, China	B	**a**	4	4B	ND	2	1	Boekhout *et al.* (1997)	EF544840	EF544786	EF211203	AJ300926‖	EF211411	EF211531	EF211651	EF211766
CBS7748 (=IFM50902)	Air in *Eucalyptus camaldulensis* hollow, Balranald, SA, Australia	B	α	4	4B	VGI	4	2	Boekhout *et al.* (1997)	EF544841	EF544787	EF211204	AJ300927‖	EF211412	EF211532	EF211652	EF211767
CBS8273 (=CBS6289, =RV20186, = NIH B-3939)	Subculture of type strain of *Cryptococcus gattii* (RV 20186)	B	**a**	4A	4	ND	2	4	Boekhout *et al.* (1997)	EF544842	EF544788	EF211205	EF211304	EF211413	EF211533	EF211653	EF211768
E566 (=CBS11233)	*E. camaldulensis* tree #19 hollow 4, Renmark Australia	B	**a**	4	4	VGI	1	2	Halliday (2000)	EF544843	EF544789	EF211206	EF211305	EF211414	EF211534	EF211654	EF211769
RV20186 (=CBS6289, =CBS8273, =NIH B-3939) (T)	Cerebrospinal fluid, type strain of *Cryptococcus gattii*, Congo (Zaire)	B	**a**	4A	4A	ND	2	4	Gatti & Eeckels (1970)	EF544844	EF544790	EF211207	EF211306	EF211415	EF211535	EF211655	EF211770

Table 2. Continued.

Isolate	Origin	Sero-type	Mating type	Nuclear genotype*	AFLP geno-type†,‡,§	Mole-cular type¶	MtLrRNA haplotype	*ATP6* haplo-type	References /sources	MtLrRNA	*ATP6*	ITS	IGS	*RPB1*	*RPB2*	*CNLAC1*	*TEF1*α
RV54130	Second isolate of *C. neoformans* var. *shanghaiensis*, China	B	**a**	4	4B	ND	2	1	Boekhout *et al*. (2001)	EF544845	EF544791	EF211208	EF211307	EF211416	EF211536	EF211656	EF211771
WM176	*Eucalyptus citriodora,* USA	B	α	4	4B	ND	2	1	Boekhout *et al*. (2001)	EF544846	EF544792	EF211209	EF211308	EF211417	EF211537	EF211657	EF211772
WM179 (R)	Immunocompetent human, reference strain of molecular type VGI, Sydney, Australia	B	α	4	4	VGI	1	2	Meyer *et al*. (2003)	EF544847	EF544793	EF211210	EF211309	EF211418	EF211538	EF211658	EF211773
WM276 (=CBS10510)	*Eucalyptus tereticornis*, Mt. Annan, NSW, Australia	B	α	4	4	VGI	2	1	Kidd *et al*. (2005)	EF544848	EF544794	EF211211	EF211310	EF211419	EF211539	EF211659	EF211774
WM830	Immunocompetent, Human, Papua. New Guinea	B	**a**	4	4	VGI	2	1	Katsu *et al*. (2004)	EF544849	EF544795	EF211212	EF211311	EF211420	EF211540	EF211660	EF211775
C. gattii AFLP5 = VGIII																	
380C	Unknown	C	α	5	5C	ND	5	5	Boekhout *et al*. (2001)	EF544850	EF544796	EF211213	EF211312	EF211421	EF211541	EF211661	EF211776
384C	Patient	C	α	5	5C	ND	5	8	Boekhout *et al*. (2001)	EF544851	EF544797	EF211214	EF211313	EF211422	EF211542	EF211662	EF211777
CBS5758	Unknown	C	α	5	5C	ND	5	5	Boekhout *et al*. (1997)	EF544852	EF544798	EF211215	AJ300929‖	EF211423	EF211543	EF211663	EF211778
CBS6955 (T) (=NIH 191)	Human, type strain of *Cryptococcus bacillisporus*, CA, USA	C	**a**	5	5C	ND	5	7	Boekhout *et al*. (1997)	EF544853	EF544799	EF211216	AJ300940‖	EF211424	EF211544	EF211664	EF211779
CBS6993 (=NIH 18)	Human, CA, USA	C	α	5	5C	ND	5	5	Boekhout *et al*. (1997)	EF544854	EF544800	EF211217	EF211314	EF211425	EF211545	EF211665	EF211780
CBS6996	Man	B	α	5	5C	ND	5	8	Boekhout *et al*. (1997)	EF544855	EF544801	EF211218	AJ300939‖	EF211426	EF211546	EF211666	EF211781
CBS8755 (=HOO58-I-682)	Detritus of almond tree, Colombia	C	α	5	5A	ND	5	5	Boekhout *et al*. (2001)	EF544856	EF544802	EF211219	EF211315	EF211427	EF211547	EF211667	EF211782
CN043 (=CBS11247)	Human, Auckland, New Zealand	B	α	5	5	VGIII	5	6	Katsu *et al*. (2004)	EF544857	EF544803	EF211220	EF211316	EF211428	EF211548	EF211668	EF211783
WM161 (R)	*Eucalyptus camaldulensis* wood from hollow, reference strain of molecular type VGIII, San Diego, CA, USA	B	α	5B	5B	VGIII	5	5	Meyer *et al*. (2003)	EF544858	EF544804	EF211221	EF211317	EF211429	EF211549	EF211669	EF211784
WM726	*Eucalyptus citriodora*, San Diego, CA, USA	B	α	5B	5B	VGIII	5	5	Boekhout *et al*. (2001)	EF544859	EF544805	EF211222	EF211318	EF211430	EF211550	EF211670	EF211785
WM728	*Eucalyptus* sp. debris from car park of zoo, San Diego, CA, USA	B	α	5B	5B	VGIII	5	5	Boekhout *et al*. (2001)	EF544860	EF544806	EF211223	EF211319	EF211431	EF211551	EF211671	EF211786

C. gattii AFLP6 = VGII																		
A1M F2866 (=CBS11240)	Dead wild Dall's porpoise lymph node, Shores of Gulf Island, Canada	B	α	6	6A	VGIIa	1	2	Kidd *et al.* (2004)	EF544861	EF544807	EF211224	EF211320	EF211432	EF211552	EF211672	EF211787
A1M F2932 (=CBS11235)	Immunocompetent male, lung, Kelowna, Canada	B	α	6	6A	VGIIa	1	2	Kidd *et al.* (2004)	EF544862	EF544808	EF211225	EF211321	EF211433	EF211553	EF211673	EF211788
A1M R265 (=CBS10514)	Immunocompetent male, Duncan, Vancouver Island, Canada	B	α	6	6A	VGIIa	1	2	Kidd *et al.* (2004)	EF544863	EF544809	EF211226	EF211322	EF211434	EF211554	EF211674	EF211789
A1M R269 (=CBS11236)	Immunocompetent female, Victoria, Canada	B	α	6	6A	VGIIa	1	2	Kidd *et al.* (2004)	EF544864	EF544810	EF211227	EF211323	EF211435	EF211555	EF211675	EF211790
A1M R271 (=CBS11237)	Immunocompetent male, Nanoose Bay, Vancouver Island, Canada	B	α	6	6A	VGIIa	1	2	Kidd *et al.* (2004)	EF544865	EF544811	EF211228	EF211324	EF211436	EF211556	EF211676	EF211791
A1M R368 (=CBS11238)	Immunocompetent male, Victoria, Canada	B	α	6	6A	VGIIa	1	2	Kidd *et al.* (2004)	EF544866	EF544812	EF211229	EF211325	EF211437	EF211557	EF211677	EF211792
A1M R406 (=CBS11239)	Immunocompetent female, Nanaimo, Vancouver Island, Canada	B	α	6	6A	VGIIa	1	2	Kidd *et al.* (2004)	EF544867	EF544813	EF211230	EF211326	EF211438	EF211558	EF211678	EF211793
A1M R409	Immunocompetent female, Victoria, Canada	B	α	6	6A	VGIIa	1	2	Kidd *et al.* (2004)	EF544868	EF544814	EF211231	EF211327	EF211439	EF211559	EF211679	EF211794
CBS1930	Sick goat, Aruba	B	**a**	6	6B	ND	1	2	Boekhout *et al.* (1997)	EF544869	EF544815	EF211232	AJ300919‖	EF211440	EF211560	EF211680	EF211795
CBS6956 (=NIH 444, =ATCC32609)	Immunocompetent human, Seattle, WA, USA	B	α	6	6A	VGII	1	2	Boekhout *et al.* (1997)	EF544870	EF544816	EF211233	AJ300920‖	EF211441	EF211561	EF211681	EF211796
CBS7750	*Eucalyptus camaldulensis* bark debris, San Francisco, CA, USA	B	α	6	6A	VGII	1	2	Boekhout *et al.* (1997)	EF544871	EF544817	EF211234	AJ300922‖	EF211442	EF211562	EF211682	EF211797
CBS8684	Nest of wasp, Uruguay	B	α	6	6B	ND	1	2	Boekhout *et al.* (2001)	EF544872	EF544818	EF211235	EF211328	EF211443	EF211563	EF211683	EF211798
HEC11102	Human, Rio de Janeiro, Brazil	B	**a**	6	6B	VGII	1	2	Katsu *et al.* (2004)	EF544873	EF544819	EF211236	EF211329	EF211444	EF211564	EF211684	EF211799
ICB184 (=MTPI2, =CBS11251)	Hollow trees, Piaui, Brazil	B	α	6	6B	ND	1	2	Barreto de Oliveira *et al.* (2004)	EF544874	EF544820	EF211237	EF211330	EF211445	EF211565	EF211685	EF211800
RAM2	*Eucalyptus camaldulensis*, Arnhemland, NT, Australia	B	α	6	6B	VGII	1	2	Katsu *et al.* (2004)	EF544875	EF544821	EF211238	EF211331	EF211446	EF211566	EF211686	EF211801
WM178 (=IFM 50894) [R]	Immunocompetent human, lung, reference strain of molecular type VGII, Sydney, Australia	B	α	6	6A	VGII	1	2	Meyer *et al.* (2003)	EF544876	EF544822	EF211239	EF211332	EF211447	EF211567	EF211687	EF211802

Table 2. Continued.

Isolate	Origin	Sero-type	Mating type	Nuclear genotype*	AFLP geno-type†,‡,§	Mole-cular type¶	MtLrRNA haplotype	*ATP6* haplo-type	References /sources	MtLrRNA	*ATP6*	ITS	IGS	*RPB1*	*RPB2*	*CNLAC1*	*TEF1*α
C. gattii AFLP7 = VGIV																	
B-5742	Human, Punjab, India	C	α	7	7	VGIV	3	9	Katsu *et al.* (2004)	EF544877	EF544823	EF211240	EF211333	EF211448	EF211568	EF211688	EF211803
B-5748	HIV positive patient, India	C	α	7	7	ND	3	9	Diaz & Fell (2005)	EF544878	EF544824	EF211241	EF211334	EF211449	EF211569	EF211689	EF211804
M27055	Clinical, Johannesburg, South Africa	C	α	7	7	VGIV	2	9	Latouche *et al.* (2002)	EF544879	EF544825	EF211242	EF211335	EF211450	EF211570	EF211690	EF211805
WM779 (=IFM50896) (R)	Cheetah, reference strain of molecular type VGIV, Johannesburg, South Africa	C	α	7	7	VGIV	2	9	Meyer *et al.* (2003)	EF544880	EF544826	EF211243	EF211336	EF211451	EF211571	EF211691	EF211806
C. neoformans var. *grubii outgroup isolates*																	
125.91 (=CBS10512)	Cryptococcal meningitis patient, Tanzania	A	**a**	1	1	ND	NA	NA	Lengeler *et al.* (2002)	EF544827	EF544773	EF211129	EF211249	EF211337	EF211457	EF211577	EF211692
H99 (=CBS8710, =CBS10515) (T)	Patient with Hodgkin's disease, type strain of *Cryptococcus neoformans* var. *grubii*, NY, USA	A	α	1	1	VNI	NA	NA	Franzot *et al.* (1999)	EF544828	EF544774	EF211146	EF211264	EF211354	EF211474	EF211594	EF211709
WM714	Cat paranasal, Australia	A	α	1B	1B	ND	NA	NA	Boekhout *et al.* (2001)	EF544829	EF544775	EF211174	EF211289	EF211382	EF211502	EF211622	EF211737
C. neoformans var. neoformans outgroup isolates																	
CBS6886 (=NIH 430)	Dropping of pigeon	D	α	2	2	ND	NA	NA	Boekhout *et al.* (1997)	EF544830	EF544776	EF211182	AJ300886‖	EF211390	EF211510	EF211630	EF211745
JEC20 (=CBS10511, =NIH-B4476)	Congenic pair with JEC21 that differs only in matingtype	D	**a**	2	2	ND	NA	NA	Kwon-Chung *et al.* (1992)	EF544831	EF544777	EF211189	EF211296	EF211397	EF211517	EF211637	EF211752

*Bovers et al. (2008c).
†Boekhout *et al*. (2001).
‡Barreto de Oliveira *et al*. (2004).
§F. Hagen, (unpublished data).
¶Meyer *et al*. (2003).
‖Diaz *et al*. (2000).
**Bovers *et al*. (2008a).
MtLrRNA and *ATP6* haplotypes are indicated for *Cryptococcus gattii* isolates and GenBank accession numbers are given for all sequenced regions.
ND, not determined; NA, not applicable; (T), type strain; (R), reference strain.

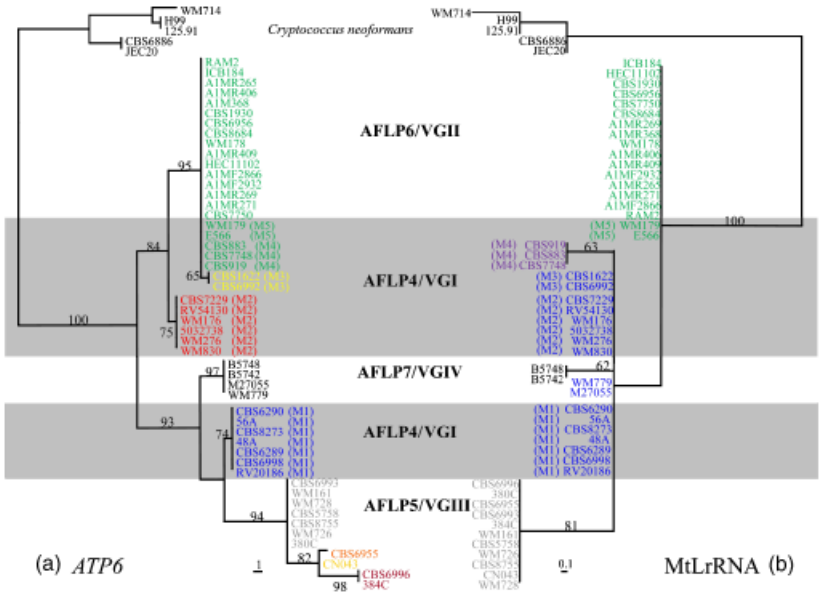

Fig. 1. Phylogenetic tree of *Cryptococcus gattii* obtained by analysis of (a) partial ATP6 sequences. Presented is one of the four most parsimonious trees (length 83, consistency index 0.819, retention index 0.974) computed with gaps treated as missing data. Data consisted of 611 characters of which 64 characters were parsimony informative; (b) partial MtLrRNA sequences. Presented is one of three most parsimonious trees (length 15, consistency index 1.000, retention index 1.000) computed with gaps treated as missing data. Data consisted of 299 characters of which 13 characters were parsimony informative. Bootstrap values (1000 replicates) are indicated for the main branches. h, haplotype number; M, mitochondrial genotype. Haplotypes of both ATP6 and MtLrRNA are indicated by the colors given in Fig. 2. In addition, those not included in Fig. 2 are as follows: ATP6 – h5, grey; h6, ochre; h7, orange; h8, brown; h9, black; MtLrRNA – h3, black; h5, grey.

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 Table 2. Three isolates (CBS6289, CBS8273 and RV20186) were independently included in the analysis, but could be traced back to the same original isolate, and accordingly were found to have the same mitochondrial haplotypes. To confirm the correctness of our sequence data, especially related to the observed chimeric sequences, single colonies were selected, and processed for DNA extraction, PCR and sequence analysis. Furthermore, in an independent research project, a number of isolates from each lineage that were included in the present study were independently processed for the analysis of *ATP6* sequences. Both gave the same sequences; hence, we are confident that we have been working with correct sequences (F. Hagen & T. Boekhout, unpublished data).

Alignment and phylogenetic analyses

The program SEQMAN 5.03 (DNASTAR, Madison, WI) was used to assemble consensus sequences and these were checked manually. Sequences were aligned using CLUSTALX version 1.8 (Thompson *et al.*, 1997) and visually corrected using GENEDOC version 2.5.000 (http//:www.nrbsc.org/downloads/). Neighbor-Joining (NJ) and Maximum Parsimony (MP) phylogenetic analyses were performed using PAUP* (PHYLOGENETIC ANALYSIS USING PARSIMONY) version 4.0b10 (Swofford, 2000). NJ analyses were carried out with the uncorrected ('p'), Jukes–Cantor, Kimura two-parameter and HKY85 substitution models. Any ties that were encountered were broken randomly. Bootstrap analysis (Hillis & Bull, 1993) with 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 as the branch-swapping algorithm. All characters were unordered and of equal weight; gaps were treated both as missing and as a new character state. Bootstrap analysis was performed with 1000 replicates.

Results

ATP6

Analysis of partial *ATP6* sequences showed that among 611 analyzed nucleotides, 34 nucleotides were polymorphic within *C. gattii*. Nine haplotypes could be identified (Fig. 1a). Haplotype 1 was present in AFLP4/VGI isolates 503 2738, CBS7229, RV54130, WM176, WM276 and WM830. All AFLP6/VGII isolates and AFLP4/VGI isolates WM179, E566, CBS883, CBS919 and CBS7748 possessed haplotype 2. Haplotype 3 was identified in AFLP4/VGI isolates CBS1622 and CBS6992. Haplotype 4 was found in AFLP4/VGI isolates 48A, 56A, CBS6289, CBS6290, CBS6998, CBS8273 and RV20186. AFLP5/VGIII isolates 380C, CBS5758, CBS6993, CBS8755, WM161, WM726 and WM728 possessed haplotype 5, whereas haplotype 6 was present in AFLP5/VGIII isolate CN043. Haplotype 7 was found in AFLP5/VGIII isolate CBS6955 and haplotype 8 was present in AFLP5/VGIII isolates 384C and CBS6996. All AFLP7/VGIV isolates possessed haplotype 9. The *ATP6* haplotype of each isolate is indicated in Fig. 1a and Table 2. The alignment of the partial *ATP6* sequences is given in Supporting Information, Fig. S1.

NJ and MP analyses showed a division of *C. gattii* into two clades. One clade consisted of haplotypes 1–3, and was found to be supported by bootstrap values ranging from 84% to 91% for NJ and MP analyses, respectively. The other clade contained haplotypes 4–9 and was found to be strongly supported by bootstrap values ranging from 93% to 99% for NJ and MP analyses. Haplotype 1 formed a separate cluster supported by bootstrap values ranging from 94% to 99% for NJ and from 75% to 76% for MP analyses. The cluster consisting of haplotypes 2 and 3 was strongly supported by bootstrap values ranging from 95% to 100% for NJ and MP analyses. Haplotype 4 formed a separate cluster supported by bootstrap values ranging from 97% to 99% for NJ and from 74% to 75% for MP analyses. Haplotypes 5–8 formed a cluster that was supported by bootstrap values ranging from 92% to 94% for NJ and MP analyses. Haplotype 5 formed a separate cluster in NJ analyses, supported by bootstrap values ranging from 87% to 97% in NJ and 94% in MP analyses. Haplotypes 6–8 also formed a strongly supported cluster with bootstrap values ranging from 93% to 95% for NJ and from 82 to 83% for MP analyses. A separate haplotype 8 cluster was strongly supported by bootstrap values ranging from 98% to 99% for NJ and MP analyses. Haplotype 9 formed a separate cluster strongly supported by bootstrap values ranging from 97% to 100%.

MtLrRNA

Analysis of partial MtLrRNA sequences resulted in the identification of five *C. gattii* haplotypes (Fig. 1b, Table 2). The alignment of the five haplotypes is shown in Fig. S2. Four polymorphic nucleotides, which are indicated within brackets, were present among 299 analyzed nucleotides. Haplotype 1 (CTAG) was present in all AFLP6/VGII isolates and in AFLP4/VGI isolates WM179 and E566; haplotype 2 (CTAC) was present in most of the AFLP4/VGI isolates and in AFLP7/VGIV isolates M27055 and WM779; haplotype 3 (CTTC) was present in AFLP7/VGIV isolates B5742 and B5748; haplotype 4 (CGAC) was present in AFLP4/VGI isolates CBS883, CBS919 and CBS7748; and haplotype 5 (GTAA) was present in all AFLP5/VGIII isolates. An overview of the MtLrRNA haplotype of all isolates is given in Table 2. Both NJ and MP analyses positioned haplotype 1 basal to the other haplotypes, but the topology of the tree of haplotypes 1–4 was not well supported. Haplotype 5 formed a separate cluster in NJ and MP analyses with bootstrap values ranging from 80% to 86%.

Combined analysis of mitochondrial data

All AFLP6/VGII isolates contained MtLrRNA haplotype 1 and *ATP6* haplotype 2, whereas AFLP7/VGIV isolates possessed MtLrRNA haplotypes 2 or 3 that differed by one nucleotide, and *ATP6* haplotype 9. The AFLP5/VGIII isolates contained MtLrRNA haplotype 5 and *ATP6* haplotypes 5–8 that formed one cluster. AFLP4/VGI isolates possessed MtLrRNA haplotypes 1–4 and *ATP6* haplotypes 1–4. Surprisingly, these haplotypes did not cluster together. The AFLP4/VGI isolates could be divided into five mitochondrial genotypes based on the presence of ATP6 and MtLrRNA alleles (viz. M1–M5). Genotype AFLP4/VGI-M1 (MtLrRNA haplotype 2 and *ATP6* haplotype 4, i.e. isolates 48A, 56A, CBS6289, CBS6290, CBS6998, CBS 8273, RV 20186) corresponded to the previously described genotypic subgroup AFLP4A (Boekhout *et al.*, 2001). This genotype formed a clade with AFLP5/VGIII and AFLP7/VGIV isolates in the *ATP6* analyses (Fig. 1a) similar to the topology obtained by analyses of six concatenated nuclear regions (see Bovers *et al.*, 2008b, Fig. 3). Genotype AFLP4/VGI-M1 thus appears to be the core AFLP4/VGI genotype, because they contain AFLP4/VGI-typical alleles of both the nuclear and mitochondrial loci, and in the phylogenetic analysis of the nuclear genes these isolates appeared basal. Genotype M2 of AFLP4/VGI (MtLrRNA haplotype 2 and *ATP6* haplotype 1, i.e. isolates 503 2738, CBS7229, RV54130, WM176, WM276 and WM830) possessed the core AFLP4/VGI MtLrRNA sequence. Interestingly, the *ATP6* sequence of this genotype, i.e. haplotype 1, was found to be a chimerical sequence in all the isolates studied. This sequence was partially identical to the sequence found in AFLP6/VGII isolates (i.e. h2) and partially identical to the sequence of isolates with a core AFLP4/VGI mitochondrial sequence (i.e. h4, Fig. S3). Genotype AFLP4/VGI-M3 (MtLrRNA haplotype 2 and *ATP6* haplotype 3, i.e. isolates CBS1622, CBS6992) possessed the core AFLP4/VGI MtLrRNA sequence and an *ATP6* sequence that differed only at one nucleotide position from the *ATP6* sequence found in AFLP6/VGII isolates. Genotype AFLP4/VGI-M4 (MtLrRNA haplotype 4 and *ATP6* haplotype 2, i.e. isolates CBS883, CBS919, CBS7748) possessed a MtLrRNA sequence that differed one nucleotide from the core AFLP4/VGI MtLrRNA sequence and an *ATP6* sequence identical to that of AFLP6/VGII isolates. Genotype AFLP4/VGI-M5 (MtLrRNA haplotype 1 and *ATP6* haplotype 2, i.e. isolates E566, WM179 possessed MtLrRNA and *ATP6* sequences identical to those found in AFLP6/VGII isolates. Isolate WM179 and isolate E566 that both belong to the mitochondrial genotype M5, have *MAT*α and *MATa*, respectively. This indicates that the presence of the AFLP6/VGII allele in AFLP4/VGI isolates does not depend on the *MAT* locus. Thus, the mitochondrial genome of part of the AFLP4/VGI isolates contained sequences that were partially identical to those found in AFLP6/VGII isolates. The incongruence between the mitochondrial genotypes of AFLP4/VGI and the topology of the AFLP4/VGI clade as derived from the analysis of six

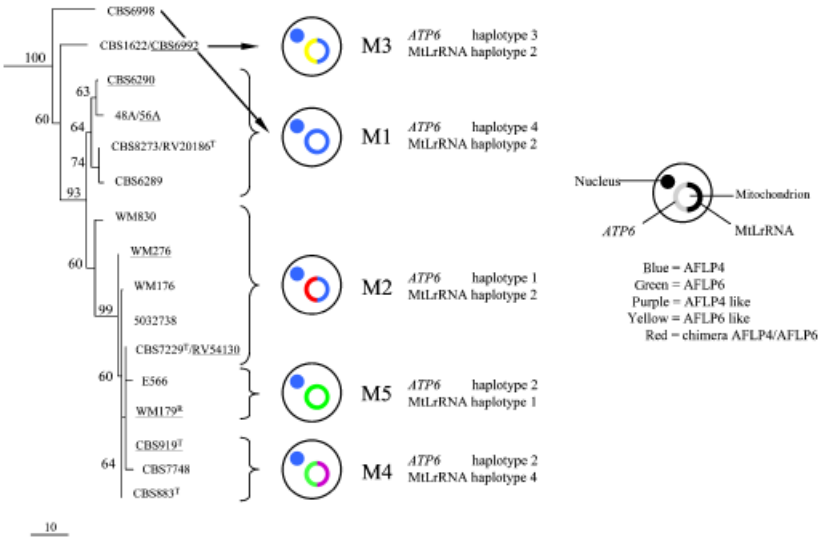

Fig. 2. Comparison of mitochondrial genotypes and a phylogenetic tree of genotype AFLP4/VGI of *Cryptococcus gattii* obtained by analysis of six concatenated nuclear regions (*RPB1*, *RPB2*, *CNLAC1*, *TEF1α*, IGS1 and internal transcribed spacer). Presented is one of the 60 most parsimonious trees (length 847, consistency index 0.902, retention index 0.961) computed with gaps treated as missing data. Data consisted of 3932 characters of which 459 characters were parsimony informative. Bootstrap values (1000 replicates) are indicated. The mitochondrial genotype as well as the *ATP6* and MtLrRNA haplotypes are shown. T, type strain; R, molecular type reference strain (Meyer *et al.*, 2003). Haploid isolates are underlined.

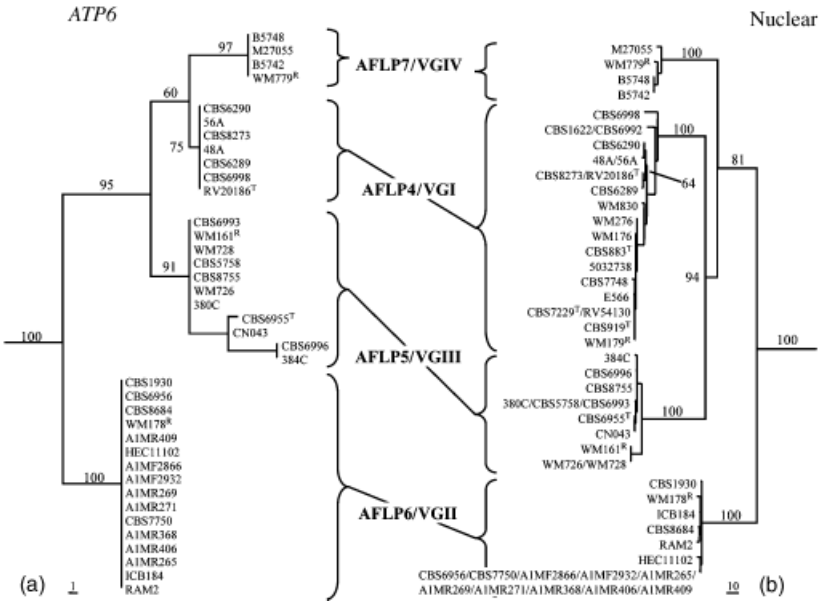

Fig. 3. Phylogenetic tree of *Cryptococcus gattii* obtained by analysis of (a) partial *ATP6* sequences. AFLP4/VGI isolates that possessed mitochondrial sequences identical to sequences found in AFLP6/VGII isolates were excluded from the analysis. Presented is the most parsimonious tree (length 79, consistency index 0.861, retention index 0.978) computed with gaps treated as missing data. Data consisted of 611 characters of which 64 characters were informative of parsimony; (b) six concatenated nuclear regions (*RPB1*, *RPB2*, *CNLAC1*, *TEF1α*, IGS1 and internal transcribed spacer). Presented is one of the 60 most parsimonious trees (length 847, consistency index 0.902, retention index 0.961) computed with gaps treated as missing data. Data consisted of 3932 characters of which 459 characters were informative of parsimony. Bootstrap values (1000 replicates) are indicated for the main branches. T, type strain; R, molecular type reference strain (Meyer *et al.*, 2003).

concatenated nuclear regions (Bovers *et al.*, 2008b) is illustrated in Fig. 2.

Phylogenetic analyses of partial *ATP6* sequences were also carried out without the presence of the AFLP4/VGI isolates that contained sequences that are partially identical to those found in AFLP6/VGII isolates. These AFLP4/VGI isolates were excluded because their presence might obscure the genotypic structure of *C. gattii*. The topology obtained is depicted in Fig. 3. The cluster that contained isolates with core AFLP4/VGI alleles and AFLP7/VGIV isolates formed a sister group to the AFLP5/VGIII isolates, and this AFLP4/VGI-AFLP7/VGIV-AFLP5/VGIII clade was strongly supported with bootstrap values ranging from 98% to 99% for NJ and from 94% to 95% for MP analyses. The AFLP6/VGII isolates clustered basal to the other *C. gattii* genotypic groups in both the mitochondrial and nuclear analyses with strong support.

Discussion

Phylogenetic analyses of partial mitochondrial sequences of *C. gattii* showed that AFLP6/VGII isolates clustered basal to all other *C. gattii* genotypic groups. These results correspond to the topology that was found after the analysis of six concatenated nuclear regions (Bovers *et al.*, 2008b; Fig. 3).

Surprisingly, two AFLP4/VGI isolates (AFLP4/VGI-M5) were found that possessed MtLrRNA and *ATP6* sequences identical to those found in AFLP6/VGII isolates. In addition, three AFLP4/VGI isolates (AFLP4/VGI-M4) possessed a MtLrRNA sequence that differed by one nucleotide from the core AFLP4/VGI sequence, but the *ATP6* sequence was identical to the sequence found in all AFLP6/VGII isolates. Furthermore, two AFLP4/VGI isolates (AFLP4/VGI-M3) possessed the core AFLP4/VGI MtLrRNA sequence, whereas the *ATP6* sequence differed by one nucleotide from the sequence found in AFLP6/VGII isolates. In addition, six AFLP4/VGI isolates (AFLP4/VGI-M2) possessed the core AFLP4/VGI MtLrRNA sequence, but here the *ATP6* sequence appeared to be a chimera between core AFLP4/VGI and AFLP6/VGII sequences.

Our results show that AFLP4/VGI isolates exist that possess mitochondrial genomes that consist completely of AFLP6/VGII sequences or that contain a combination of AFLP4/VGI and AFLP6/VGII sequences. It could be argued that the presence of AFLP6/VGII mitochondrial sequences in AFLP4/VGI isolates is a retained ancestral character state, because genotype AFLP6/VGII was found to be the ancestral lineage (Bovers *et al.*, 2008b). However, in that case AFLP6/VGII mitochondrial sequences would be expected to occur in other genotypic groups of *C. gattii* as well, especially in AFLP7/VGIV as this genotypic group is most closely related to AFLP6/VGII (Bovers *et al.*, 2008b). We have to keep in mind, however, that the number of available AFLP7/VGII isolates is small (i.e. only four strains are currently know to belong to this group); thus, it is possible that nucleotide variation in this lineage remains to be detected in the future when more isolates will become available. In the case that ancestral mitochondrial sequences remained preserved in the more derived lineages, viz., AFLP7/VGIV, AFLP5/VGIII and AFLP4/VGI, one would expect that the same or similar sequences would be present in the phylogenetically intermediate genotypes because the patterns of divergence of the various genotypic groups is well supported by a phylogenetic analysis of six nuclear genes (Bovers *et al.*, 2008b). Moreover, one would expect that some polymorphisms would be present due to ongoing evolution since the initial divergence of the various genotypic groups of *C. gattii*. In contrast with these suppositions, we observed only AFLP4/VGI isolates that possessed *ATP6* sequences identical to those found in AFLP6/VGII isolates. These sequences did not occur in any of the other, phylogenetically intermediate, genotypes, viz., AFLP7/VGIV and AFLP5/VGIII. This suggests that the presence of AFLP6/VGII mitochondrial sequences in AFLP4/VGI isolates is not a retained ancestral character state but, more likely, is a consequence of mitochondrial recombination. The likelihood of mitochondrial recombination is further supported by the presence of the MtLrRNA haplotype 2 allele of AFLP4/VGI in two isolates of AFLP7/VGIV. If this would be the result of retaining ancestral sequences, this process would contradict the well-supported phylogeny based on six nuclear genes in which genotype AFLP4/VGI is more derived than AFLP7/VGIII (Bovers *et al.*, 2008b).

AFLP4/VGI and AFLP6/VGII isolates have been isolated from the same geographic regions (Sorrell *et al.*, 1996; Kidd *et al.*, 2003, 2005, 2007; Campbell *et al.*, 2005a, b), which indicates that these genotypic groups occur in the same areas and may physically interact. Interestingly, AFLP4/VGI isolates that possessed mitochondrial genomes with (partial) AFLP6/VGII sequences were all isolated from countries near the Pacific Ocean, whereas isolates with core AFLP4/VGI sequences originated from Africa and Europe. Although the number of isolates included in our study is small, this may indicate that the atypical AFLP4/VGI isolates originated in the region of the Pacific Ocean.

If mitochondrial recombination has occurred, one has to assume a process of somatic fusion or mating to occur between cells of the recombining genotypes. To the best of our knowledge, somatic fusion without mating has never been reported in *C. neoformans* or in *C. gattii*. Mating usually occurs between isolates of opposite mating type (*MAT*a and *MAT*α) (Kwon-Chung, 1975, 1976a, b), but has also been reported between isolates of identical mating type (Lin *et al.*, 2005). During mating, the nucleus of the *MAT*α cell migrates into the conjugation tube and the recipient *MAT*a cell generates a hypha (McClelland *et al.*, 2004). Previous reports indicated that mitochondria in *C. neoformans* were uniparentally inherited from the *MAT*a parent (Xu *et al.*, 2000; Yan & Xu, 2003), probably because the *MAT*a cell produces the hypha. However, recent studies have shown that in some cases mitochondrial inheritance from the *MAT*α may occur as well (Yan & Xu, 2003; Toffaletti *et al.*, 2004; Yan *et al.*, 2004, 2007a, b) resulting in the presence of *MAT*α parental mitochondria in the progeny. Recent studies on other fungal species have shown that strictly uniparental or biparental mitochondrial inheritance is rare (Yan & Xu, 2005), and it has therefore been suggested to treat mitochondrial inheritance as a quantitative rather than a qualitative trait (Birky, 1995). In cells of the mitochondrial genotype M5, which combine a nuclear genome of the genotype AFLP4/VGI with the presence of the mitochondrial alleles *ATP6* and MtLrRNA from AFLP6/VGII, cells of both *MATa* (i.e. isolate E566) and *MAT*α (i.e. isolate WM179) seem to have functioned as a donor for mitochondrial genes. Therefore, in these cases, the transfer of mitochondria does not seem to be regulated by the mating type. Mitochondrial inheritance following AFLP4/VGI × AFLP6/VGII mating may have resulted in a cell with both types of mitochondria. Subsequent mitotic divisions and mitochondrial selection could have resulted in isolates that possessed a complete AFLP6/VGII mitochondrial

genome. In addition, recombination between the two types of mitochondria that were present may have resulted in mitochondrial genomes with both AFLP4/VGI and AFLP6/VGII sequences. It is important to note that recombination between mitochondria has been observed in other fungi under laboratory conditions, for example *Agaricus bisporus* (De la Bastide & Horgen, 2003), *Agrocybe aegerita* (Barroso & Labarère, 1997), *Neurospora intermedia* (Yang & Griffiths, 1992), *Saccharomyces cerevisiae* (Nakagawa *et al*., 1992), as well as in natural populations, for example *A. aegerita* (Barroso *et al*., 1995), *Armillaria gallica* (Saville *et al*., 1998), *Candida albicans* (Anderson *et al*., 2001; Jacobsen *et al*., 2008) and *Neurospora crassa* (Taylor *et al*., 1986). In addition, mitochondrial recombination has been observed in laboratory crossings of *C. neoformans* isolates (Toffaletti *et al*., 2004; Yan *et al*., 2007b).

Here we suggest the occurrence of mitochondrial recombination among environmental, veterinary and clinical isolates of *C. gattii*. Recombination between two mitochondrial genomes from different genotypes is only possible after cell–cell conjugation occurs. However, in that case one might expect the occurrence of hybrid nuclear genomes within the populations, and this is not the case with the loci that we have studied (Bovers *et al*., 2008b). Hybrid isolates between the two *C. neoformans* varieties and between *C. neoformans* and *C. gattii* have been described (Tanaka *et al*., 1999; Cogliati *et al*., 2000; Boekhout *et al*., 2001; Bovers *et al*., 2006, 2008c), as has DNA exchange between serotype A and serotype D isolates of *C. neoformans* (Kavanaugh *et al*., 2006). The resulting hybrid isolates are diploid or aneuploid (Tanaka *et al*., 1999; Cogliati *et al*., 2000; Lengeler *et al*., 2001; Bovers *et al*., 2006, 2008c) and possess alleles from both parents. However, all AFLP4/VGI isolates that had been studied using flow cytometry were found to be haploid (M. Bovers & H. Hoogveld, unpublished data) and nuclear AFLP6/VGII sequences have not been found in these AFLP4/VGI isolates (Bovers *et al*., 2008b), which indicates that the isolates, most likely, are not hybrids. Possibly, mating between different *C. gattii* genotypic groups does not result in the formation of hybrid nuclear genomes, but may lead to transfer of mitochondria and subsequent mitochondrial recombination. Moreover, the predominant uniparental inheritance of mitochondria as well as the small size of the mitochondrial genome increases the chance of incorporation of foreign alleles by drift and selection (Martinsen *et al*., 2001; Funk & Omland, 2003; Ballard & Whitlock, 2004). The predominant uniparental inheritance and the haploid nature of mitochondria both decrease the effective population size and thereby increase the chance of fixation by drift (Funk & Omland, 2003; Ballard & Whitlock, 2004). In addition, the small genome size decreases the probability of counter selection because negative gene interactions are less likely to occur (Martinsen *et al*., 2001; Ballard & Whitlock, 2004). These processes could explain why mitochondrial regions, but not nuclear regions, were incorporated in a different genetic background. Another possibility is that smaller regions of the AFLP6/VGII genome have been incorporated in parts of the nuclear genome of AFLP4/VGI isolates, but that these have escaped detection.

Mitochondria play a central role in energy production and in responses to stresses (Osiewacz & Kimpel, 1999; Ahkter *et al*., 2003). These processes play a key role in the fitness of an organism, and the mitochondrial genotype may therefore influence fitness as well. A different mitochondrial genotype may have a positive effect on fitness, as was observed in *S. cerevisiae* when mitochondria of wine yeasts were transferred to a laboratory yeast, resulting in an increased viability and increased ethanol and temperature tolerance (Jiménez & Benítez, 1988). However, the mitochondrial genotype does not always affect fitness, and no difference in virulence or high-temperature growth rate could be found for strains with mitochondria derived from both *C. neoformans* varieties (Toffaletti *et al*., 2004). In addition, the mitochondrial genotype may also have a negative effect on fitness. Several mitochondrial proteins are produced in the nucleus (Osiewacz & Kimpel, 1999; Burton *et al*., 2006) and these nuclear encoded proteins interact with mitochondrial encoded proteins, for example in the electron transport system. The presence of a mitochondrial genotype different from the nuclear genotype may therefore negatively affect fitness as both genomes have coevolved (Burton *et al*., 2006). *Cryptococcus gattii* isolates with different mitochondrial genotypes should be further studied to investigate whether the mitochondrial genotype affects their fitness.

Our results indicate that conjugation may have occurred between AFLP4/VGI and isolates of both AFLP6/VGII and AFLP7/VGIV leading to the generation of cells with an AFLP4/VGI nuclear genotype and a partial AFLP6/VGII or AFLP7/VGIV mitochondrial genotype. Our study is the first to report on mitochondrial inheritance in *C. gattii* and provides evidence for mitochondrial recombination occurring in nature. Furthermore, the high percentage of AFLP4/VGI isolates studied that possessed a recombined mitochondrial genome (65%) indicates that recombination occurs frequently in nature. Recently, recombination has been demonstrated to occur in natural populations of genotype AFLP4/VGI of *C. gattii* using AFLP analysis (Saul *et al*., 2008). Future research is needed to elucidate the genetic mechanism of the intergenotypic transfer of mitochondria in *C. gattii* and the effect this has on phenotypic properties. The occurrence of mitochondrial recombination between isolates of genotypes AFLP4/VGI and AFLP6/VGII may also be important, because strains of the latter are involved in the ongoing Vancouver Island outbreak of *C. gattii* (Kidd *et al*.,

2004; MacDougall *et al.*, 2007). Plasmogamy between cells belonging to different genotypes may allow the exchange and introgression of genetic elements in either genetic background, as has also been observed between isolates of serotype A and serotype D of *C. neoformans* (Kavanaugh *et al.*, 2006), and such processes may contribute to the formation of *C. gattii* strains that differ in virulence. It is likely that such genetic transfer events may have an effect on the virulence properties, and this alternative hypothesis concurs with the same sex mating hypothesis (Fraser *et al.*, 2005; Lin *et al.*, 2005) that has been proposed to explain the origin of hypervirulence of isolates involved in the ongoing Vancouver Island outbreak of *C. gattii*.

Acknowledgements

Isolates were kindly donated by the following researchers: J.W. Kronstad, K.J. Kwon-Chung, M.S. Lazera, D. Swinne, W. Meyer and J.M. Torres-Rodríguez. We would like to thank Cañan Bayrakdar and Hans Hoogveld for technical assistance. Work of M.B. was supported by the 'Odo van Vloten fonds'. E.E.K. and F.H. were funded by the Strategy Fund of the Royal Netherlands Academy of Arts and Sciences (RNAAS-KNAW).

Authors' contributions

M.B. and F.H. equally contributed to the work, M.B. and F.H. performed the work, T.B. designed the research, and M.B., F.H., E.E.K. and T.B. wrote the paper.

References

Ahkter S, McDade HC, Gorlach JM, Heinrich G, Cox GM & Perfect JR (2003) Role of alternative oxidase gene in pathogenesis of *Cryptococcus neoformans*. *Infect Immun* **71**: 5794–5802.

Anderson JB, Wickens C, Khan M, Cowen LE, Federspiel N, Jones T & Kohn LM (2001) mtDNA recombination in a natural population. *J Bacteriol* **183**: 865–872.

Ballard JWO & Whitlock MC (2004) The incomplete natural history of mitochondria. *Mol Ecol* **13**: 729–744.

Barreto de Oliveira MT, Boekhout T, Theelen B, Hagen F, Baroni FA, Lazera MS, Lengeler KB, Heitman J, Rivera ING & Paula CR (2004) *Cryptococcus neoformans* shows a remarkable genotypic diversity in Brazil. *J Clin Microbiol* **42**: 1356–1359.

Barroso G & Labarère J (1997) Genetic evidence for nonrandom sorting of mitochondria in the basidiomycete *Agrocybe aegerita*. *Appl Environ Microbiol* **63**: 4686–4691.

Barroso G, Blesa S & Labarere J (1995) Wide distribution of mitochondrial genome rearrangements in wild strains of the cultivated basidiomycete *Agrocybe aegerita*. *Appl Environ Microbiol* **61**: 1187–1193.

Birky CW (1995) Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *P Natl Acad Sci USA* **92**: 11331–11338.

Biswas SK, Wang L, Yokoyama K & Nishimura K (2003) Molecular analysis of *Cryptococcus neoformans* mitochondrial cytochrome *b* gene sequences. *J Clin Microbiol* **41**: 5572–5576.

Boekhout T, van Belkum A, Leenders ACAP, Verbrugh HA, Mukamurangwa P, Swinne D & Scheffers WA (1997) Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects. *Int J Syst Bacteriol* **47**: 432–442.

Boekhout T, Theelen B, Diaz M, Fell JW, Hop WCJ, Abeln ECA, Dromer F & Meyer W (2001) Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology* **147**: 891–907.

Bolano A, Stinchi S, Preziosi R, Bistoni F, Allegrucci M, Baldelli F, Martini A & Cardinali G (2001) Rapid methods to extract DNA and RNA from *Cryptococcus neoformans*. *FEMS Yeast Res* **1**: 221–224.

Bovers M, Hagen F, Kuramae EE, Diaz MR, Spanjaard L, Dromer F, Hoogveld HL & Boekhout T (2006) Unique hybrids between fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*. *FEMS Yeast Res* **6**: 599–607.

Bovers M, Hagen F & Boekhout T (2008a) Diversity of the *Cryptococcus neoformans–Cryptococcus gattii* species complex. *Rev Iberoam Mycol* **25**: S4–S12.

Bovers M, Hagen F, Kuramae EE & Boekhout T (2008b) Six monophyletic lineages identified within *Cryptococcus neoformans* and *Cryptococcus gattii* by multi-locus sequence typing. *Fungal Genet Biol* **45**: 400–421.

Bovers M, Hagen F, Kuramae EE, Hoogveld HL, Dromer F, St-Germain G & Boekhout T (2008c) AIDS patient death caused by novel *Cryptococcus neoformans* × *C. gattii* hybrid. *Emerg Infect Dis* **14**: 1105–1108.

Burton RS, Ellison CK & Harrison JS (2006) The sorry state of F_2 hybrids: consequences of rapid mitochondrial DNA evolution in allopatric populations. *Am Nat* **168**: S14–S24.

Butler MI & Poulter RTM (2005) The *PRP8* inteins in *Cryptococcus* are a source of phylogenetic and epidemiological information. *Fungal Genet Biol* **42**: 452–463.

Callejas A, Ordóñez N, Rodriguez MC & Castañeda E (1998) First isolation of *Cryptococcus neoformans* var. *gattii*, serotype C, from the environment in Colombia. *Med Mycol* **36**: 341–344.

Campbell LT, Currie BJ, Krockenberger M, Malik R, Meyer W, Heitman J & Carter D (2005a) Clonality and recombination in genetically differentiated subgroups of *Cryptococcus gattii*. *Eukaryot Cell* **4**: 1403–1409.

Campbell LT, Fraser JA, Nichols CB, Dietrich FS, Carter D & Heitman J (2005b) Clinical and environmental isolates of *Cryptococcus gattii* from Australia retain sexual fecundity. *Eukaryot Cell* **4**: 1410–1419.

Chaturvedi V, Fan J, Stein B, Behr MJ, Samsonoff WA, Wickes BL & Chaturvedi S (2002) Molecular genetic analyses of mating pheromones reveal intervariety mating or hybridization in *Cryptococcus neoformans*. *Infect Immun* **70**: 5225–5235.

Cogliati M, Allaria M, Liberi G, Tortorano AM & Viviani MA (2000) Sequence analysis and ploidy determination of *Cryptococcus neoformans* var. *neoformans*. *J Mycol Med* **10**: 171–176.

De la Bastide PY & Horgen PA (2003) Mitochondrial inheritance and the detection of non-parental mitochondrial DNA haplotypes in crosses of *Agaricus bisporus* homokaryons. *Fungal Genet Biol* **38**: 333–342.

Diaz MR & Fell JW (2005) Use of a suspension array for rapid identification of the varieties and genotypes of the *Cryptococcus neoformans* species complex. *J Clin Microbiol* **43**: 3662–3672.

Diaz MR, Boekhout T, Theelen B & Fell JW (2000) Molecular sequence analyses of the intergenic spacer (IGS) associated with rDNA of the two varieties of the pathogenic yeast, *Cryptococcus neoformans*. *Syst Appl Microbiol* **23**: 535–545.

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

Ellis D, Marriott D, Hajjeh RA, Warnock D, Meyer W & Barton R (2000) Epidemiology: surveillance of fungal infections. *Med Mycol* **38**: 173–182.

Ellis DH & Pfeiffer TJ (1990) Natural habitat of *Cryptococcus neoformans* var. *gattii*. *J Clin Microbiol* **28**: 1642–1644.

Escandón P, Sánchez A, Martínez M, Meyer W & Castañeda E (2006) Molecular epidemiology of clinical and environmental isolates of the *Cryptococcus neoformans* species complex reveals a high genetic diversity and the presence of the molecular type VGII mating type a in Colombia. *FEMS Yeast Res* **6**: 625–635.

Fell JW, Boekhout T, Fonseca A, Scorzetti G & Statzell-Tallman A (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**: 1351–1371.

Fortes ST, Lazéra MS, Nishikawa MM, Macedo RCL & Wanke B (2001) First isolation of *Cryptococcus neoformans* var. *gattii* from a native jungle tree in the Brazilian Amazon rainforest. *Mycoses* **44**: 137–140.

Franzot SP, Salkin IR & Casadevall A (1999) *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. *J Clin Microbiol* **37**: 838–840.

Fraser JA, Subaran RL, Nichols CB & Heitman J (2003) Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii:* implications for an outbreak on Vancouver Island, Canada. *Eukaryot Cell* **2**: 1036–1045.

Fraser JA, Giles SS, Wenink EC *et al.* (2005) Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature* **437**: 1360–1364.

Funk DJ & Omland KE (2003) Species-level paraphyly and polyphyly: frequency, causes and consequences, with insights from animal mitochondrial DNA. *Annu Rev Ecol Evol S* **34**: 397–423.

Gatti F & Eeckels R (1970) An atypical strain of *Cryptococcus neoformans* (Sanfelice) Vuillemin 1894. Part 1. Description of the disease and of the strain. *Ann Soc Belg Med Tr* **50**: 689–693.

Granados DP & Castañeda E (2005) Isolation and characterization of *Cryptococcus neoformans* varieties recovered from natural sources in Bogotá, Colombia and study of ecological conditions in the area. *Microbial Ecol* **49**: 282–290.

Halliday CL (2000) A molecular study of mating type and recombination in *Cryptococcus neoformans* var. *gattii*. PhD Thesis, University of Sydney, Sydney, Australia.

Hillis DM & Bull JJ (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst Biol* **42**: 182–192.

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

Jacobsen MD, Rattray AMJ, Gow NAR, Odds FC & Shaw DJ (2008) Mitochondrial haplotypes and recombination in *Candida albicans*. *Med Mycol* **46**: 647–654.

Jiménez J & Benítez T (1988) Yeast cell viability under conditions of high temperature and ethanol concentrations depends on the mitochondrial genome. *Curr Genet* **13**: 461–469.

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

Kavanaugh LA, Fraser JA & Dietrich FS (2006) Recent evolution of the human pathogen *Cryptococcus neoformans* by intervarietal transfer of a 14 gene fragment. *Mol Biol Evol* **23**: 1879–1890.

Kidd SE, Sorrell TC & Meyer W (2003) Isolation of two molecular types of *Cryptococcus neoformans* var. *gattii* from insect frass. *Med Mycol* **41**: 171–176.

Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, MacDougall L, Boekhout T, Kwon-Chung KJ & Meyer W (2004) A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Colombia, Canada). *P Natl Acad Sci USA* **101**: 17258–17263.

Kidd SE, Guo H, Bartlett KH, Xu J & Kronstad JW (2005) Comparative gene genealogies indicate that two clonal lineages of *Cryptococcus gattii* in British Columbia resemble strains from other geographical areas. *Eukaryot Cell* **4**: 1629–1638.

Kidd SE, Chow Y, Mak S, Bach PJ, Chen H, Hingston AO, Kronstad JW & Bartlett KH (2007) Characterization of environmental sources of the human and animal pathogen *Cryptococcus gattii* in British Columbia, Canada, and Pacific Northwest USA. *Appl Environ Microbiol* **73**: 1433–1443.

Krockenberger MB, Canfield PJ & Malik R (2002) *Cryptococcus neoformans* in the koala (*Phascolarctos cinereus*): colonization by *C. neoformans* var. *gattii* and investigation of environmental sources. *Med Mycol* **40**: 263–272.

Kwon-Chung KJ (1975) A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. *Mycologia* **67**: 1197–1200.

Kwon-Chung KJ (1976a) Morphogenesis of *Filobasidiella neoformans*, the sexual state of *Cryptococcus neoformans*. *Mycologia* **68**: 821–833.

Kwon-Chung KJ (1976b) A new species of *Filobasidiella*, the sexual state of *Cryptococcus neoformans* B and C serotypes. *Mycologia* **68**: 942–946.

Kwon-Chung KJ & Bennett JE (1984) Epidemiological differences between the two varieties of *Cryptococcus neoformans*. *Am J Epidemiol* **120**: 123–130.

Kwon-Chung KJ & Varma A (2006) Do major species concepts support one, two or more species within *Cryptococcus neoformans*? *FEMS Yeast Res* **6**: 574–587.

Kwon-Chung KJ, Bennett JE & Rhodes JC (1982) Taxonomic studies on *Filobasidiella* species and their anamorphs. *Antonie van Leeuwenhoek* **48**: 25–38.

Kwon-Chung KJ, Edman JC & Wickes BL (1992) Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infect Immun* **60**: 602–605.

Kwon-Chung KJ, Boekhout T, Fell JW & Diaz M (2002) Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacillisporus* (*Basidiomycota*, *Hymenomycetes*, *Tremellomycetidae*). *Taxon* **51**: 804–806.

Latouche GN, Sorrell TC & Meyer W (2002) Isolation and characterisation of the phospholipase B gene of *Cryptococcus neoformans* var. *gattii*. *FEMS Yeast Res* **2**: 551–561.

Latouche GN, Huynh M, Sorrell TC & Meyer W (2003) Isolation and characterisation of the phospholipase B gene of *Cryptococcus neoformans* var. *gattii*. *Appl Environ Microbiol* **69**: 2080–2086.

Lazéra MS, Cavalcanti MA, Trilles L, Nishikawa MM & Wanke B (1998) *Cryptococcus neoformans* var. *gattii* – evidence for a natural habitat related to decaying wood in a pottery tree hollow. *Med Mycol* **36**: 119–122.

Lazéra MS, Salmito Cavalcanti MA, Londero AT, Trilles L, Nishikawa MM & Wanke B (2000) Possible primary ecological niche of *Cryptococcus neoformans*. *Med Mycol* **38**: 379–383.

Lengeler KB, Cox GM & Heitman J (2001) Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. *Infect Immun* **69**: 115–122.

Lengeler KB, Wang P, Cox GM, Perfect JR & Heitman J (2002) Identification of the *MAT*a mating-type locus of *Cryptococcus neoformans* reveals a serotype A *MAT*a strain thought to have been extinct. *P Natl Acad Sci USA* **97**: 14455–14460.

Lin X, Hull CM & Heitman J (2005) Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans*. *Nature* **434**: 1017–1021.

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

MacDougall L, Kidd SE, Galanis E, Mak S, Leslie MJ, Cieslak PR, Kronstad JW, Morshed MG & Bartlett KH (2007) Spread of *Cryptococcus gattii* in British Columbia, Canada, and detection in the Pacific Northwest, USA. *Emerg Infect Dis* **13**: 42–50.

Martinsen GD, Whitham TG, Turek RJ & Keim P (2001) Hybrid populations selectively filter gene introgression between species. *Evolution* **55**: 1325–1335.

McClelland CM, Chang YC, Varma A & Kwon-Chung KJ (2004) Uniqueness of the mating system in *Cryptococcus neoformans*. *Trends Microbiol* **12**: 208–212.

Meyer W, Castañeda A, Jackson S, Huynh M & Castañeda Eand the IberoAmerican Cryptococcal, study, group (2003) Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis* **9**: 189–195.

Nakagawa K, Morishima N & Shibata T (1992) An endonuclease with multiple cutting sites, Endo.SceI, initiates genetic recombination at its cutting site in yeast mitochondria. *EMBO J* **11**: 2707–2715.

Osiewacz HD & Kimpel E (1999) Mitochondrial-nuclear interactions and lifespan control in fungi. *Exp Gerontol* **34**: 901–909.

Randhawa HS, Kowshik T & Khan ZU (2003) Decayed wood of *Syzygium cumini* and *Ficus religiosa* living trees in Delhi/New Delhi metropolitan area as natural habitat of *Cryptococcus neoformans*. *Med Mycol* **41**: 199–209.

Ruma P, Chen SC, Sorrell TC & Brownlee AG (1996) Characterization of *Cryptococcus neoformans* by random DNA amplification. *Lett Appl Microbiol* **23**: 312–316.

Saul N, Krockenberger M & Carter D (2008) Evidence of recombination in mixed-mating-type and alpha-only populations of *Cryptococcus gattii* sourced from single eucalyptus tree hollows. *Eukaryot Cell* **7**: 727–734.

Saville BJ, Kohli Y & Anderson JB (1998) mtDNA recombination in a natural population. *P Natl Acad Sci USA* **95**: 1331–1335.

Sorrell TC, Brownlee AG, Ruma P, Malik R, Pfeiffer TJ & Ellis DH (1996) Natural environmental sources of *Cryptococcus neoformans* var. *gattii*. *J Clin Microbiol* **34**: 1261–1263.

Speed B & Dunt D (1995) Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans*. *Clin Infect Dis* **21**: 28–34.

Stephen C, Lester S, Black W, Fyfe M & Raverty S (2002) Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. *Can Vet J* **43**: 792–794.

Swofford DL (2000) *PAUP* 4.0: Phylogenetic Analysis Using Parsimony.* Sinauer Associates, Sunderland, MA.

Tanaka R, Nishimura K & Miyaji M (1999) Ploidy of serotype AD strains of *Cryptococcus neoformans*. *Jpn J Med Mycol* **40**: 31–34.

Taylor JW, Smolich BD & May G (1986) Evolution and mitochondrial DNA in *Neurospora crassa*. *Evolution* **40**: 716–739.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**: 4876–4882.

Toffaletti DL, Nielsen K, Dietrich F, Heitman J & Perfect JR (2004) *Cryptococcus neoformans* mitochondrial genomes from serotype A and D strains do not influence virulence. *Curr Genet* **46**: 193–204.

Upton A, Fraser JA, Kidd SE, Bretz C, Bartlett KH, Heitman J & Marr KA (2007) First contemporary case of human infection with *Cryptococcus gattii* in Puget Sound: evidence for spread of the Vancouver Island outbreak. *J Clin Microbiol* **45**: 3086–3088.

Viviani MA, Cogliati M, Esposto MC, Lemmer K, Tintelnot K, Colom MF, Swinnen D, Velegraki A & Velho Rand European Confederation of medical Mycology. (2006) *Cryptococcus neoformans* isolates from a 30-month ECMM survey of cryptococcosis in Europe. *FEMS Yeast Res* **6**: 614–619.

White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (Innis MA, Gelfand DH, Sninsky JJ & White TJ, eds), pp. 315–322. Academic Press, San Diego.

Xu J, Ali RY, Gregory DA, Amick D, Lambert SE, Yoell HJ, Vilgalys RJ & Mitchell TG (2000) Uniparental mitochondrial transmission in sexual crosses in *Cryptococcus neoformans*. *Curr Microbiol* **40**: 269–273.

Yan Z & Xu J (2003) Mitochondria are inherited from the *MAT*a parent in crosses of the basidiomycete fungus *Cryptococcus neoformans*. *Genetics* **163**: 1315–1325.

Yan Z & Xu J (2005) Fungal mitochondrial inheritance and evolution. *Evolutionary Genetics of Fungi* (Xu J, ed), pp. 221–252. Horizon Bioscience, Norfolk, UK.

Yan Z, Hull CM, Heitman J, Sun S & Xu J (2004) *SXI1*α controls uniparental mitochondrial inheritance in *Cryptococcus neoformans*. *Curr Biol* **14**: 743–744.

Yan Z, Hull CM, Sun S, Heitman J & Xu J (2007a) The mating type-specific homeodomain genes *SXI1*α and *SXI2a* coordinately control uniparental mitochondrial inheritance in *Cryptococcus neoformans*. *Curr Genet* **51**: 187–195.

Yan Z, Sun S, Shahid M & Xu J (2007b) Environment factors can influence mitochondrial inheritance in the fungus *Cryptococcus neoformans*. *Fungal Genet Biol* **44**: 315–322.

Yang X & Griffiths AJF (1992) Male transmission of linear plasmids and mitochondrial DNA in the fungus *Neurospora*. *Genetics* **134**: 1055–1062.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of *Cryptococcus gattii* partial *ATP6* sequences.

Fig. S2. Alignment of *Cryptococcus gattii* partial MtLrRNA sequences.

Fig. S3. Alignment of *Cryptococcus gattii* partial *ATP6* sequences for haplotypes 1, 2 and 4.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.