M13 PCR fingerprinting detects genetic instability of *Cryptococcus gattii* after passage through a rat model of infection

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Abstract

Cryptococcus gattii is a basidiomycetous yeast causing infections in humans and animals mainly in immonocompetent hosts. In *Cryptococcus neoformans*, genetic adaptation to environmental conditions has been shown by several studies and lead to phenotypic changes to benefit survival of the yeast. However, none of the studies focused on *C. gattii*. In the current study, we investigated the occurrence of genome changes in isolates of *C. gattii* molecular type VGII, which were obtained after passage through a rat model of infection. Serial isolates from four original strains were studied for genetic variation as detected by M13 PCR fingerprinting. Most of the studied strains showed a high genetic stability except one veterinary isolate, McBride, for which three distinct PCR-fingerprinting patterns were observed. Our study provides basic evidence of genetic adaptation of *C. gattii* in response to host environmental stresses.

Key words: *Cryptococcus gattii*, genetic instability, M13 PCR fingerprinting, animal passage.

Introduction

Cryptococcus gattii, previously known as *Cryptococcus neoformans* var. *gattii* is a basidiomycetous yeast causing life-threatening mycoses in humans and animals mainly in immunocompetent hosts (Kwon-Chung *et al.* 2002; Sorrell 2001). Target organs of the yeast are the central nervous system and lungs, but also other parts of the body can be affected, such as skin, prostate glands, urinary tract, eyes, myocardium, bones and joint (Casadevall and Perfect 1998). *C. gattii* are saprophytic yeasts colonizing the bark of a variety of trees species including *Eucalyptus* species (Ellis and Pfeiffer, 1990) and *Laurus* species (Escandon *et al.* 2006). Up to date, four major molecular types, VGI, VGII, VGIII and VGIV, have been designated to this species (Meyer *et al.* 2003). The VGI molecular type is the most common causing infections worldwide. However, the VGII molecular type has gained importance as being the causative agent of a recent ongoing outbreak of cryptococcosis on Vancouver Island, British Columbia, Canada (Kidd *et al.* 2004).

The pathogenesis of cryptococcosis, an infection caused by this yeast, is still controversial. However, it is proposed that the yeast enters the host either as dehydrated haploid yeast or as basidiospore via the respiratory tract. After a certain period of incubation in the lungs, it spreads to extrapulmonary tissue, especially the central nervous system, via the blood stream. If untreated, the infection can be fatal (Lin and Heitman 2006).

To be pathogenic, an adaptation to new environments is indispensable in order to withstand a variety of stresses as possessed by the host immune response, Re-assortment of genetic elements after encountering such defensive mechanism is reported in several pathogenic organisms especially viruses (Urwin *et al.* 2004; Wei *et al.* 2006; Chen *et al.* 2008). However, such events are complicated and can take much longer time to occur in eukaryotic genomes such as protozoa and fungi (Dewar and Bernier 1995; David Sibley 2003; Fraser *et al.* 2004). In *Cryptococcus neoformans*, the closest sibling species of *C. gattii*, some papers reported genetic stability, in which the karyotypes of individual isolates remained stable during both passage in a rabbit model (Perfect *et al.* 1993) and infections in humans (Sukroongreung *et al.* 2001; Sullivan *et al.* 1996). In both studies, only minor polymorphisms were detected. On the contrary, there is a study from B. Fries *et al.,* which showed changes in the karyotype in sequential isolates obtained during infection (Fries *et al*. 1996). None of those studies have investigated *C. gattii*. Hence, we conducted an experiment to describe the genetic stability of *C. gattii* strains during passage through a rat model of infection, using M13 PCR fingerprinting based genotyping.

Materials and Methods

Strains and media

Serial isolates of four strains of the VGII molecular type recovered from the lungs of infected rats (Krockenberger et al. unpublished data) at different time points were used in this study (Table 1). In addition, the eight molecular type standard strains for the *Cryptococcus* species complex were used as a reference strains: WM 148 (serotype A, VNI/AFLP1), WM 626 (serotype A, VNII/AFLP1A), WM 628 (serotype AD, VNIII/AFLP2), WM629 (serotype D, VNIV/AFLP3), WM 179 (serotype B, VGI/AFLP4), WM 178 (serotype B, VGII/AFLP6), WM 175 (serotype B, VGIII/AFLP5), and WM 779 (serotype C, VGIV/AFLP7) (Boekhout *et al.*, 2001; Meyer *et al.*, 2003) (Table 1). All strains were retrieved from the culture collection of the Molecular Mycology Research Laboratory, Centre for Infectious Disease and Microbiology, Westmead Hospital, Sydney, Australia. All strains were subcultured on Sabouraud Dextrose Agar (2% glucose, 1% peptone and 2% agar) before undergoing DNA extraction.

DNA extraction

DNA was extracted from the cultures as described previously with minor modifications (Ferrer *et al.*, 2001). Briefly, about half of an inoculation loop of cell culture was transferred into a microcentrifuge tube and stored at -20°C for at least 30 minutes.

Table 1 Strain information.

Strain name	WM Number	Source	Country	Mating type	Days after inoculation/ organs isolated from/ Rat number	References
R265	WM 02.32	Clinical	Canada	α	0 (original isolate)	Kidd *et al.* 2004
R265B22-1	WM 06.52	Clinical	Canada	α	22/brain/1	
R265LV22-1	WM 06.53	Clinical	Canada	α	22/liver/1	
R265L22-1	WM 06.54	Clinical	Canada	α	22/lung/1	
R265L58-3	WM 06.48	Clinical	Canada	α	58/lung/3	
R265L58-4	WM 06.49	Clinical	Canada	α	58/lung/4	
R272	WM 02.39	Clinical	Canada	α	0 (original isolate)	Kidd *et al.* 2004
R272L22-1	WM 06.55	Clinical	Canada	α	22/lung/1	
R272L22M-1*	WM 06.56*	Clinical	Canada	α	22/lung/1	
R272L58-3	WM 06.46	Clinical	Canada	α	58/lung/3	
McBride	WM 03.312	Veterinary	Australia	α	0 (original isolate)	Kluger *et al.* 2006
McBrideL22-1	WM 06.58	Veterinary	Australia	α	22/lung/1	
McBrideL58-3	WM 06.40	Veterinary	Australia	α	58/lung/3	
McBrideB58-3	WM 06.41	Veterinary	Australia	α	58/brain/3	
McBrideL58-4	WM 06.42	Veterinary	Australia	α	58/lung/4	
McBrideB58-4	WM 06.43	Veterinary	Australia	α	58/brain/4	
McBrideLV58-4	WM 06.47	Veterinary	Australia	α	58/liver/4	
WM05.336	WM 05.336	Environment	Colombia	α	0 (original isolate)	Escandon *et al.* 2006
336L22-1	WM 06.59	Environment	Colombia	α	22/lung/1	
336L22M-1*	WM 06.60*	Environment	Colombia	α	22/lung/1	
336LV22-1	WM 06.61	Environment	Colombia	α	22/liver/1	
336L58-4	WM 06.44	Environment	Colombia	α	58/lung/4	
336S58-4	WM 06.45	Environment	Colombia	α	58/spleen/4	
336B58-4	WM 06.50	Environment	Colombia	α	58/brain/4	
336LV58-4	WM 06.51	Environment	Colombia	α	58/liver/4	

*indicates strains with changes in morphology from dry to mucous colonies.

After incubation time, 500 µL lysis buffer solution (for 100 mL solution: 0.5 g SDS, 1.4 g NaCl, 0.73 g EDTA, 20 mL Tris-HCL) as well as 5 µL 2-Mercaptoethanol were added. The tubes were vortexed vigorously and incubated at 65°C for one hour. Samples were vortexed during incubation time at least once. Afterwards, DNA was purified with 500 µL phenol: chloroform: isoamyl alcohol (v:v:v 25:24:1). DNA was precipitated with isopropanol, pelleted and washed once with 200 µL of 70% aqueous solution of ethanol. The DNA pellet was then resuspended in 50 µL distilled water. The DNA concentration was determined using a spectrophotometer. Subsequently, the DNA was diluted (10 ng/µL) for PCR.

PCR fingerprinting (M13) for fungal cultures

The PCR fingerprinting was done as described previously (Meyer *et al.*, 1999). Briefly, the diluted DNA (2.5 µL of 10 ng/µL) was mixed with 47.5 µL of PCR master mix [5 µL of 10 X PCR buffer, 5 µL of 2 mM dNTPs, 3 µL of 50 mM magnesium acetate, 3 µL of 10 ng/µL M13-primer (5' GAGGGTGGCGGTTCT 3'), 0.5 µL AmpliTaq (Applied Biosystem, CA, USA) and 31 µL water]. The PCR amplification was performed in Perkin Elmer PCR machine (PerkinElmer Inc., MA, USA) as followed: 35 cycles of denaturation at 94ºC for 1 minute, annealing at 50ºC for 1 minute and extension of DNA at 72ºC for 20 seconds; and a final extension at 72ºC for 6 minutes. The amplicons were then visualized on 1.4% agarose gel under UV light after running at 70 V to 14 cm. After a complete run, a picture was taken and the bands were analysed using the 1D gel analysis module (BioGalaxy) implemented in the BioloMICS software ver. 7.5.88 (BioAware, Belgium) using UPGMA.

Results

Most of the strains gave PCR fingerprinting patterns identical to the patterns of the original isolates before animal passage as they clustered with 100% similarity across all isolates in each strain group (Figs 1 and 2). However, the isolates of the McBride strain surprisingly gave three different patterns after the animal passage, including the original pattern: pattern 1, pattern 2 and 3 (Figs 1 and 2). In addition, mix patterns were found in the same rat when inoculated with the strain McBride. Both patterns 1 and 3 were found in rat number 3 and 4 (Figs 1 and 2).

Discussion

It has long been known that genetic traits constantly change in response to external stimuli allowing for a phenotypic adaptation to certain environmental conditions. In *Cryptococcus neoformans*, the phenotypic adaptation has been reported in several studies which revealed major phenotypic changes (Franzot *et al.* 1998) and ultimately virulence alteration (Fries and Casadevall 1998). However, despite phenotypic adaptation, serial isolates collected from the same patient with cryptococcosis generally revealed a very high genetic stability as has been found using karyotyping (Perfect *et al.* 1993) or DNA fingerprinting (Sukroongreung *et al.* 2001; Sullivan *et al.* 1996). However, major changes have been reported in one study investigating serial isolates which have been obtained during infection (Fries

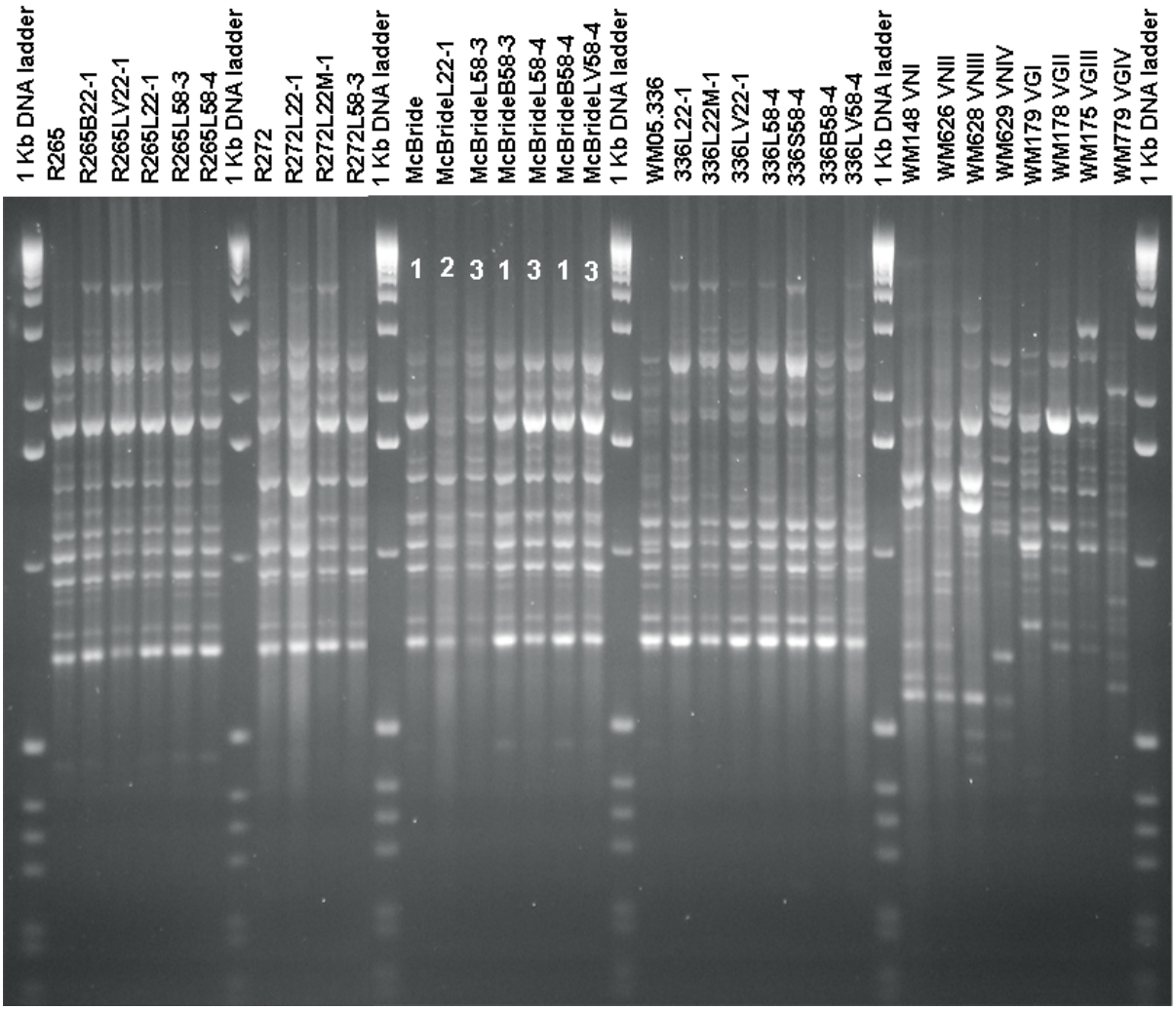

Fig. 1 Electrophoresis gel of PCR fingerprinting with the primer M13 for fungal cultures for all strains including the standards and the strains before and after inoculations. The original strains (R265, R272, McBride and WM05.336) were loaded next to the isolates obtained after animal passage and separated from each other by the molecular marker. Strains 148 (VNI), 626 (VNII), 628 (VNIII), 629 (VNIV), 179 (VGI), 178 (VGII), 175 (VGIII) and 779 (VGIV) were used as standards for comparison. 3 patterns were recognized among McBride isolates.

et al. 1996) and minor polymorphisms have occurred in some other cases (Sullivan *et al.* 1996; Sukroongreung *et al.* 2001; Almeida *et al.* 2007).

Our study also revealed in most cases a high genetic stability of the *C. gattii* VGII strains during animal passage. Most isolates were genetically stable as depicted by their identical M13 PCR fingerprinting patterns across all strains originating from the same original isolates despite the passage through the rat model. However, the McBride isolates gave three distinct patterns despite the strain had been previously shown to have a very high genetic stability using multilocus microsatellite typing (Kluger *et al.* 2006). In the present study, pattern 2 was found only once indicated that the genetic adaptation occurs in several stages in this specific case, as pattern 2 (obtained at day 22 after animal inoculation) formed a link between the original pattern 1 (obtained at day 0 before animal inoculation) and pattern 3 (obtained at day 58 after animal inoculation).

Our study has shown that, in general, *C. gattii* strains are stable during animal passage changes, but genetic changes can also occur as a result of animal passage. As such, our study provides a first evidence for genetic adaptation to the host environment for *C. gattii*. M13 PCR fingerprinting patterns are stable enough to be a good molecular tool to investigate genotype stability and to identify genome changes when isolates are passed through a strong stress situation, such as an infection in mammals. However, beside the fact that PCR fingerprinting is usually relative reproducible within a laboratory (Meyer *et al.* 1999), it is necessary to exclude potential artificial differences obtained in PCR fingerprinting due to a number if influencing factors. For that reason, further genetic and phenotypic analyses of the isolates obtained from the McBride strain after animal passage are warranted to confirm the observed genetic adaptation.

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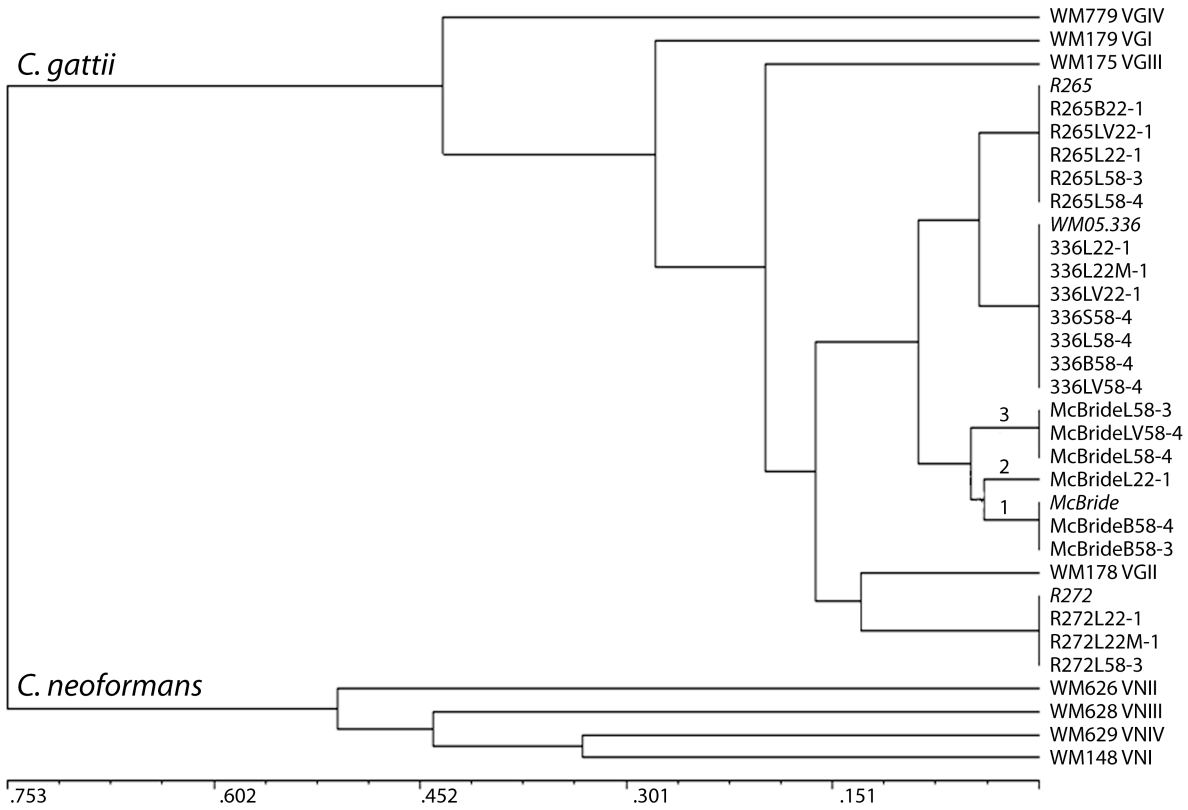

Fig. 2 Dendrogram generated using UPGMA and Dice coefficients within the BioloMICS software revealed a high genetic stability despite passages through the rat model of infection. Three patterns were recognized among the McBride isolates obtained after animal passage. (*italic = original isolates*).

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