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FEMS Yeast Research 4 (2004) 377–388

FEMS Yeast Research

www.fems-microbiology.org

The internal transcribed spacers and 5.8S rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* species complex

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Received 8 April 2003; received in revised form 28 July 2003; accepted 8 August 2003

First published online 18 September 2003

Abstract

Sequences of the internal transcribed spacer (ITS) region including the 5.8S rRNA gene delineated seven genotypes within the three varieties of *Cryptococcus neoformans* via specific combinations of eight nucleotide differences located at positions 10, 11, 15, 19, 108 (ITS1), 221 (5.8S), 298 and 346 (ITS2). The ITS types correlated to polymerase chain reaction fingerprint/random amplification of polymorphic DNA (RAPD) molecular types: with ITS type 1 (ATACTAGC) = *C. neoformans* var. *grubii*, molecular types VNI+VNII and the serotype A allele of the AD hybrid, VNIIIA; ITS type 2 (ATATAGGC) = the serotype D allele of the AD hybrid, VNIIIB, and *C. neoformans* var. *neoformans*, VNIV; and ITS type 3 (GCGCTGGC) and ITS type 7 (ACGCTGGC) = VGI = RAPD type III, ITS type 4 (ACACTGAC) = VGII = RAPD type II, ITS type 5: (ACACTGGG) = VGIII = RAPD type I, ITS type 6 (ACACTGGC) = VGIV = RAPD type IV, all corresponding to *C. neoformans* var. *gattii*. Cloned sequences from serotype AD revealed that the hybrid serotype is diploid at the ITS1-5.8S-ITS2 locus carrying the ITS type 1 (ATACTAGC) and the ITS type 2 (ATATAGGC) alleles. ITS sequencing is a useful technique for genotyping the three *C. neoformans* varieties and for subtyping within *C. neoformans* var. *gattii*.

Keywords: *Cryptococcus neoformans*; Internal transcribed spacer rDNA sequencing; Molecular typing; Random amplification of polymorphic DNA; Polymerase chain reaction fingerprinting

1. Introduction

Cryptococcus neoformans (*C.n.*) is a pathogenic fungus with three varieties currently recognized: *C.n.* var. *grubii* (serotype A), *C.n.* var. *neoformans* (serotype D), and *C.n.* var. *gattii* (serotypes B and C) [1–4]. In addition there exists a hybrid of *C.n.* var. *grubii* and *C.n.* var. *neoformans*, serotype AD. It has recently been proposed that *C. neoformans* be recognized as two distinct species, *C. neoformans* (serotypes A, AD, and D) and *C. gattii* (serotypes B and C) [5]. However, since this proposal was made after this study was finished, we will refer to three varieties of a single species, *C. neoformans*. *C.n.* var. *grubii* has recently been introduced due to genetic characteristics that distinguish it from *C.n.* var. *neoformans* [1,6]. It is the most common cause of fungal meningitis in immunocompromised hosts worldwide, and has a global distribution [1,6]. Furthermore, it has been recently reported that the prevalence of *C.n.* var. *neoformans* (serotype D) is significantly higher than previously perceived [1]. In contrast, the distribution of human cryptococcosis due to *C.n.* var. *gattii* is mainly restricted to tropical and subtropical climates, and typically afflicts immunocompetent hosts [2,7]. However, a recent outbreak of *C.n.* var. *gattii* among residents and companion animals of Vancouver Island, British Columbia, on the west coast of Canada, indicates changing patterns of distribution [8].

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1567-1356/03/$22.00
doi:10.1016/S1567-1356(03)00176-4

Table 1
List of *C. neoformans* strains used in this study and their serotype, origin, RAPD profile, ITS sequence type, PCR fingerprint molecular type and EMBL and DDBJ accession numbers

Isolate	Other collection number	Serotype	Origin	RAPD profile[a]	ITS type[b]	PCR fingerprint molecular type[c]	EMBL and DDBJ accession number
Cryptococcus neoformans var. *grubii*							
ATCC90112♦		A	Human, CSF, Pennsylvania, USA		1	VNI	AJ493550
WM148♦		A	Human, CSF, Sydney, Australia		1	VNI	AJ493551
WM626♦		A	Human, CSF, Sydney, Australia		1	VNII	AJ493552
Hamden C3′1♦		A	Pigeon dropping, Belo Horizonte, Brazil		1	VNII	AJ493553
RV58146♦		A	Wood, Zaire		1	VNII	AJ493554
IFM40216	ATCC 90113	A	Human, USA		1		AB034643
IFM5817	CUH-16	A	Human, Chiba, Japan		1		AB087812
IFM5854	CDC 551	A	Unknown, CDC, USA		1		AB087813
IFM46501	37-518-157	A	Human, NIH, Thailand		1		AB087814
IFM46554	C13	A	Human, Costa Rica		1		AB087815
IFM47273		A	Human, China		1		AB087816
Serotype AD hybrid							
RKI-M1644/90♦		AD	Human, Germany		2	VNIII	AJ493555
RKI-M364/98♦		AD	Human, Germany		2	VNIII	AJ493556
RKI-M3006/94♦		AD	Human, Germany		2	VNIII	AJ493557
WM628♦		AD	Human, CSF, Melbourne, Australia		2	VNIII	AJ493558
IFM5889	UFPR 2536	AD	Unknown, Recife, Brazil		2		AB087818
IFM45730	I-3a	AD	Pigeon dropping, China		2		AB087819
IFM45751	II-2c	AD	Pigeon dropping, China		2		AB087820
IFM46127	73-4	AD	Unknown		2		AB087824
IFM46138	CBS132	AD	Fermenting fruit juice, Italy		2	VNIII	AB087825
IFM46140	MCP 9124	AD	Unknown, Japan		2		AB087826
Cryptococcus neoformans var. *neoformans*							
B-3501♦		D	Human, CSF, Richmond, USA		2	VNIV	AJ493559
RKI-M318/90♦		D	Human, Germany		2	VNIV	AJ493560
WM629♦		D	Human, blood, Melbourne, Australia		2	VNIV	AJ493561
IFM5881	UFPR 2475	D	Unknown, Recife, Brazil		2		AB087817
IFM46081	AS 128DSL	D	Unknown, Italy		2		AB087821
IFM46084	AS 134MD	D	Unknown, Italy		2		AB087822
IFM46093	M11	D	Unknown, Italy		2		AB087823
Cryptococcus neoformans var. *gattii*							
503 2738♦		B	Human, Papua New Guinea		3	VGI	AJ493562
WM830♦		B	Human, Papua New Guinea		3	VGI	AJ493563
IFM50893	WM 179u	B	Human, CSF, Sydney, Australia		3	VGI	AB087664 AJ493564
IFM5880	UFPR 2262	B	Human, Recife, Brazil	III	3		AB087629
IFM45678	856/439	B	Unknown	III	3		AB087633
IFM45699		B	Human, Taiwan		3		AB087635
IFM45713	S-D2d, LY-6	B	Human, China	III	3		AB087636
IFM45714	S-D2y, LY-7	B	Human, China	III	3		AB087637
IFM50897	WGH-1	B	Human, Sydney, Australia	III	3	VGI	AB087668
IFM50898	W-27	B	Human, Sydney, Australia	III	3	VGI	AB087669
IFM50899	W-35	B	Human, Sydney, Australia	III	3	VGI	AB087670
IFM50900	CBS7523	B	*Eucalyptus camaldulensis* debris, Nuriootpa, Australia	III	3	VGI	AB087671
IFM50901	CBS7747	B	Olive seedling underneath *Eucalyptus camaldulensis*, Barossa Reservoir, Australia	III	3	VGI	AB087672
IFM50902	CBS7748♦	B	*Eucalyptus camaldulensis* hollow, Balranald, SA, Australia	III	3	VGI	AB087673 AJ493565
IFM50903	CBS7749	B	*Eucalyptus camaldulensis* bark, Balranald, SA, Australia	III	3	VGI	AB034647
IFM50904	WM 718♦ 413/GC2E(2)	B	*Eucalyptus tereticornis*, Gold Coast, QLD, Australia	III	3	VGI	AB087674 AJ493566
IFM5815		B	Human, Japan (Patient lived in Singapore)	III	7	VGI	AB087623
IFM46640	NACR 3	B	Unknown, Nagasaki, Japan	III	7	VGI	AB087639

Table 1 (*Continued*).

Isolate	Other collection number	Serotype	Origin	RAPD profile[a]	ITS type[b]	PCR fingerprint molecular type[c]	EMBL and DDBJ accession number
IFM48219	A465	B	Human, Parana, Brazil		7	VGI	AB087651
CBS6998	NIH 365	B	Human, CSF, Thailand	III	7		AB087657
CBS8273	NIH B-3939	B	Human, brain lesion, Africa	III	7		AB034648
TH0545		B	Human, Thailand		7	VGI	AB087675
TH0886		B	Human, Thailand		7	VGI	AB087676
HEC11102♦		B	Human, urine, Rio de Janeiro, Brazil		4	VGII	AJ493569
IFM50894	WM 178♦	B	Human, lung, Sydney, Australia		4	VGII	AB087665 AJ493567
RAM2♦		B	*Eucalyptus camaldulensis*, Arnhemland, NT, Australia		4	VGII	AJ493568
IFM5838		B	Unknown, Emory University, USA		4		AB034645
IFM5851		B	Human, USA (a subculture of NIH 444)	II	4		AB087624
IFM5883	UFPR 2527	B	Human, Recife, Brazil	II	4		AB087631
IFM46566	C61	B	Human, Costa Rica	II	4		AB087638
IFM46914	37-40-40	B	Human, NIH, Thailand	II	4		AB087640
IFM46915	37-140-140	B	Human, NIH, Thailand	II	4		AB087641
IFM46916	37-362-28	B	Human, NIH, Thailand	II	4		AB087642
IFM46917	37-381-47	B	Human, NIH, Thailand	II	4		AB087643
IFM46918	37-1852-23	B	Human, NIH, Thailand	II	4		AB087644
IFM46919	38-1539-59	B	Human, NIH, Thailand	II	4		AB087645
IFM47258	F-1623	B	Human, Funabashi, Japan (Patient born in Brazil)	II	4		AB087646
IFM48213	FT 84	B	Human, Parana, Brazil	II	4		AB087647
IFM48214	FT 78	B	Unknown, Parana, Brazil		4		AB087648
IFM48216	FT 119	B	Human, Parana, Brazil	II	4		AB087649
IFM48218	BV 33	B	Human, Venezuela	II	4		AB087650
IFM48221	A 288	B	Human, Minas Gerais, Brazil	II	4		AB087652
IFM48222	FT 102	B	Human, Parana, Brazil		4		AB087653
IFM48297	FT 130	B	Human, Parana, Brazil	II	4		AB087654
IFM48302	BV 5	B	Human, Venezuela		4		AB087655
CBS6956	NIH 444	B	Human, sputum, Washington, USA	II	4	VGII	AB087656
CBS7750♦		B	*Eucalyptus camaldulensis* bark debris, San Francisco, USA	II	4	VGII	AB087658 AJ493570
CN11		B	Human, Campinas, Brazil	II	4		AB087659
CN15		B	Human, Campinas, Brazil	II	4		AB087660
CN25		B	Human, Campinas, Brazil	II	4		AB087661
CN30		B	Human, Campinas, Brazil		4		AB087662
CN36		B	Human, Campinas, Brazil	II	4		AB087663
WM728♦		B	*Eucalyptus* sp. debris, San Diego, USA		5	VGIII	AJ493571
WM726♦		B	*Eucalyptus citriodora*, San Diego, USA		5	VGIII	AJ493572
CN043♦		B	Human, CSF, Auckland, New Zealand		5	VGIII	AJ493573
WM161♦		B	Tree hollow, San Diego, USA		5	VGIII	AJ549318
CBS6993	NIH 18	C	Human, CSF, CA, USA	I	5		AB034644
NIH112		B	Unknown, USA	I	5		AB087622
IFM5873		C	Unknown, Brazil		5		AB087625
IFM5875	UFPR-D1	C	Unknown, Recife, Brazil	I	5		AB087626
IFM5878	ENCB 1010 G-1	B	Human, Mexico	I	5		AB087627
IFM5879	ENCB 1010 G-2	B	Human, Mexico	I	5		AB087628
IFM5882	UFPR 2526	C	Unknown, Recife, Brazil	I	5		AB087630
IFM5884	UFPR 2528-1	B	Human, Recife, Brazil	I	5		AB034646
IFM5885	UFPR 2528-2	B	Human, Recife, Brazil	I	5		AB087632
IFM50895		B	Human, Melbourne, Australia	I	5	VGIII	AB087666
B-5742♦		C	Human, CSF, Punjab, India	IV	6	VGIV	AJ493574
IFM50896	WM 779♦	C	Cheetah, Johannesburg, South Africa	IV	6	VGIV	AB087667 AJ493575
Outgroups							
Cryptococcus luteolus							
CBS943♦	WM775		Air, Tokyo, Japan				AJ493576
Cryptococcus curvatus							
CBS570♦	WM56		Human, sputum, The Netherlands				AJ493577

Table 1 (*Continued*).

Isolate	Other collection number	Serotype	Origin	RAPD profile[a]	ITS type[b]	PCR fingerprint molecular type[c]	EMBL and DDBJ accession number
Cryptococcus laurentii							
ICPMR156-0720♦	WM601		Human, Sydney, Australia				AJ493578

T = type culture.

[a]RAPD pattern obtained with primer A-1 in Japan [16].

[b]ITS types 5′→3′: 1 = ATACTAGC; 2 = ATATAGGC; 3 = GCGCTGGC; 4 = ACACTGAC; 5 = ACACTGGG; 6 = ACACTGGC; 7 = ACGCTGGC (sequence position 10, 11, 15, 19, 108 (ITS1), 221 (5.8S rRNA gene), 298 and 346 (ITS2); ♦ ITS sequences are determined in Australia using primers SR6R and LR1 [20], all other ITS sequences are determined in Japan using primers ITS4 and ITS5 [18].

[c]Molecular types obtained by PCR fingerprinting with primer M13 (5′-GAGGGTGGCGGTTCT-3′) in Australia [12,13].

Several molecular typing methods such as randomly amplified polymorphic DNA (RAPD) analysis [9–11] and polymerase chain reaction (PCR) fingerprinting [12–16] have been used to characterize the genotypes of *C. neoformans*. Previous studies have sub-classified *C. neoformans* into eight molecular types, based upon PCR fingerprinting with mini- or microsatellite-specific primers [12,14,16]. Similarly, RAPD analysis has grouped *C.n.* var. *gattii* isolates into three subtypes [17]. The comparable genotypes are: *C.n.* var. *grubii* (serotype A = molecular types VNI and VNII), the serotype AD hybrid (= molecular type VNIII), *C.n.* var. *neoformans* (serotype D = molecular type VNIV), and *C.n.* var. *gattii* (serotypes B and C = molecular types VGI = RAPD type III, VGII = RAPD type II, VGIII = RAPD type I, and VGIV) with no specific correlation to serotype. The RAPD types have been correlated to internal transcribed spacer (ITS) sequence types for a limited number of *C.n.* var. *gattii* isolates in a previous study [17].

We now extend our analysis of the ITS1-5.8S-ITS2 region to all three varieties of *C. neoformans*, including 96 isolates representing all eight molecular types previously observed by PCR fingerprinting [14], in order to further characterize these molecular types and to evaluate the usefulness of ITS sequencing as a subtyping method.

2. Materials and methods

2.1. C. neoformans *strains used in the study*

Ninety-six isolates were studied in this report: 11 strains of *C.n.* var. *grubii*, seven strains of *C.n.* var. *neoformans*, 10 strains of the serotype AD hybrid and 68 strains of *C.n.* var. *gattii*. The strains were obtained either from the culture collection of the Institute of Food Microbiology (IFM) (Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan), or the Australian Medical Fungal Collection (AMFC) (Molecular Mycology Laboratory, Center for Infectious Diseases and Microbiology, ICPMR, Westmead Hospital, Westmead, NSW, Australia). See Table 1 for strain numbers and details of origin. In addition, three different *Cryptococcus* species were used as outgroups in this study: *C. luteolus* (CBS943), *C. curvatus* (CBS570) and *C. laurentii* (WM601). Fungal strains were inoculated onto potato dextrose agar (Difco, Detroit, MI, USA) slants at 30°C for approximately 48–72 h. Following two subcultures, the serotype of each strain was determined by the slide agglutination test (IATRON Crypto Check, Iatron, Tokyo, Japan) (for results see Table 1).

2.2. Extraction of DNA

DNA extractions were carried out independently in Japan or Australia using different extraction protocols. DNA extractions in Japan were carried out according to the procedures described by Tamura et al. [18,19]: 250 μl of GPT reagent (6 M guanidine thiocyanate, dissolved in 50 mM Tris pH 8.3) and 450 μl of Tris (pH 8.0)-buffered phenol were added to a suspension of washed yeast cells in a microfuge tube and the mixture was boiled for 15 min; 250 μl of chloroform–isoamyl alcohol was then added, and the aqueous phase separated by centrifugation at 12 000 × *g*, mixed with an equal amount of 100% isopropanol and 1/10 volume of 3 M sodium acetate, and placed at −20°C for 1 h. Samples were centrifuged at 12 000 × *g* for 20 min. The nucleic acid pellet was then washed with ice-cold 70% ethanol, dried and resuspended in sterile TE buffer at a concentration of 5 μg ml^{-1}. DNA extractions undertaken in Australia were carried out as previously described [13].

2.3. RAPD analysis

RAPD analysis was undertaken in Japan. The RAPD reactions were performed in a 25-μl volume containing 12.5 ng of genomic DNA, 20 pmol of primer A-1 (5′-ATT GCG TCC A-3′) [17–19], and one PCR bead (Ready-to-Go PCR bead, Amersham Pharmacia Biotech, Piscataway, NJ, USA). An initial denaturation of 94°C for 4 min was followed by 35 cycles of 94°C for 2 min, 32°C for 2 min, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. All reaction products were characterized by electrophoresis on 1.5% agarose gels in 1 × TBE buffer at 80 V for 90 min, and then stained in a

0.5 μg ml^{-1} ethidium bromide solution, and visualized by UV transillumination.

2.4. PCR fingerprinting

PCR fingerprinting analysis was undertaken in Australia. Molecular types for many of the strains included in this study have been established in a global molecular epidemiology study [12,14,16]. These data were generated according to a previously described protocol [14] and molecular types were assigned based upon comparisons to an established set of standard molecular type strains: WM148 (serotype A, VNI), WM626 (serotype A, VNII), WM628 (serotype AD, VNIII), WM629 (serotype D, VNIV), WM179 (serotype B, VGI), WM178 (serotype B, VGII), WM161 (serotype B, VGIII), and WM779 (serotype C, VGIV) [12,14,16].

2.5. ITS1-5.8S-ITS2 sequencing

Amplification of the ITS1-5.8S-ITS2 fragment was performed either in Japan or in Australia, using slightly different protocols. For the Japanese protocol, amplification reactions were performed in 30-μl volumes containing 12.5 ng of genomic DNA, 20 pmol each of primers ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) [20] and one PCR bead (Ready-to-Go PCR bead, Amersham). An initial denaturation at 94°C for 4 min was followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min [18]. The PCR products were purified using a PCR product pre-sequencing kit (USB, Cleveland, OH, USA), and then sequenced directly using a Big Dye terminator reagent kit, including *Taq* DNA polymerase, according to the protocol recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed on a 310 automated DNA sequencer (Applied Biosystems).

For the Australian protocol, amplification reactions were performed in a 50-μl volume, containing 30 ng of genomic DNA, 1×PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 0.2 mM each of dATP, dCTP, dGTP and dTTP, 3 mM magnesium acetate, 25 ng each of primers SR6R (5′-AAG $TA^{A}{}_{G}$ AAG TCG TAA CAA GG-3′) and LR1 (5′-GGT TGG TTT CTT TTC CT-3′) [21] and 2.5 U *Taq* DNA polymerase (Applied Biosystems). An initial denaturation at 97°C for 3 min was followed by 20 cycles of 97°C for 35 s, 50°C for 55 s, and 72°C for 45 s+4 s/cycle. This was followed by 10 cycles of 97°C for 45 s, 50°C for 55 s, and 72°C for 2 min+4 s/cycle, and finally 72°C for 6 min. PCR products were purified using the GeneClean Spin kit (Bio101, Quantum Technologies, Carlsbad, CA, USA) and 100 ng of the purified DNA was used to sequence both directions of the amplified fragment on an ABI 373 automated DNA sequencer (Applied Biosystems) or on a LiCor automated DNA sequencer (LiCor, Lincoln, NE, USA).

2.6. Cloning of ITS1-5.8S-ITS2 sequences from serotype AD

In light of the observations of multiple alleles at the *URA5* locus in serotype AD [21], the serotype AD ITS1-5.8S-ITS2 regions were cloned and sequenced to determine whether multiple alleles also existed at this locus. A 10-μl ligation reaction containing 1×Rapid ligation buffer (Promega, Madison, WI, USA), 50 ng pGEM cloning vector (Promega), 40 ng of purified PCR product, 3 U T4 DNA ligase was incubated at room temperature for 3 h and then stored at 4°C overnight. 6 μl of ligation reaction was combined with 40 μl of competent *Escherichia coli* JM109 cells, incubated on ice for 20 min, followed by 42°C for 2 min, and again on ice for 5 min. 950 μl SOC medium (20 g l^{-1} Bacto-tryptone (Oxoid, Basingstoke, UK), 5 g l^{-1} Bacto-yeast extract (Oxoid), 10 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, and 20 mM glucose) was added to the transformation mixture and incubated at 37°C for 1 h with shaking. 200 μl of transformed cells were spread on to LB agar containing 50 mg l^{-1} ampicillin that had been coated with 40 mg ml^{-1} of X-gal (Promega) and 100 mg IPTG (Promega). The plates were incubated at 37°C overnight. White colonies were picked from the agar with a sterile toothpick and used as the template in a PCR to re-amplify the ITS region. Amplified ITS1-5.8S-ITS2 fragments were purified and sequenced as described above.

2.7. Sequence editing and phylogeny

Sequence alignment was performed in Japan using the CLUSTALW software [23], while in Australia sequences were manually aligned using the program SeqPup v.0.6 (iubio.bio.Indiana.edu/soft/molbio/seqpup). Phylogenetic relationships between representative sequences of each ITS type were estimated by maximum-parsimony analysis using the software PAUP* 4.0b10 (PPC/Altivec) for Macintosh (Swofford 1998, Florida State University, Miami, FL, USA). The search for the most parsimonious trees was performed using the heuristic search option of PAUP* 4.0b10. Bootstrap analysis with 100 bootstrap replications using the heuristic search option of the program PAUP* 4.0b10 was carried out to test the robustness of the internal branches.

3. Results

3.1. ITS sequence signatures to subtype the three varieties of C. neoformans

ITS sequences, including the 5.8S gene, were determined

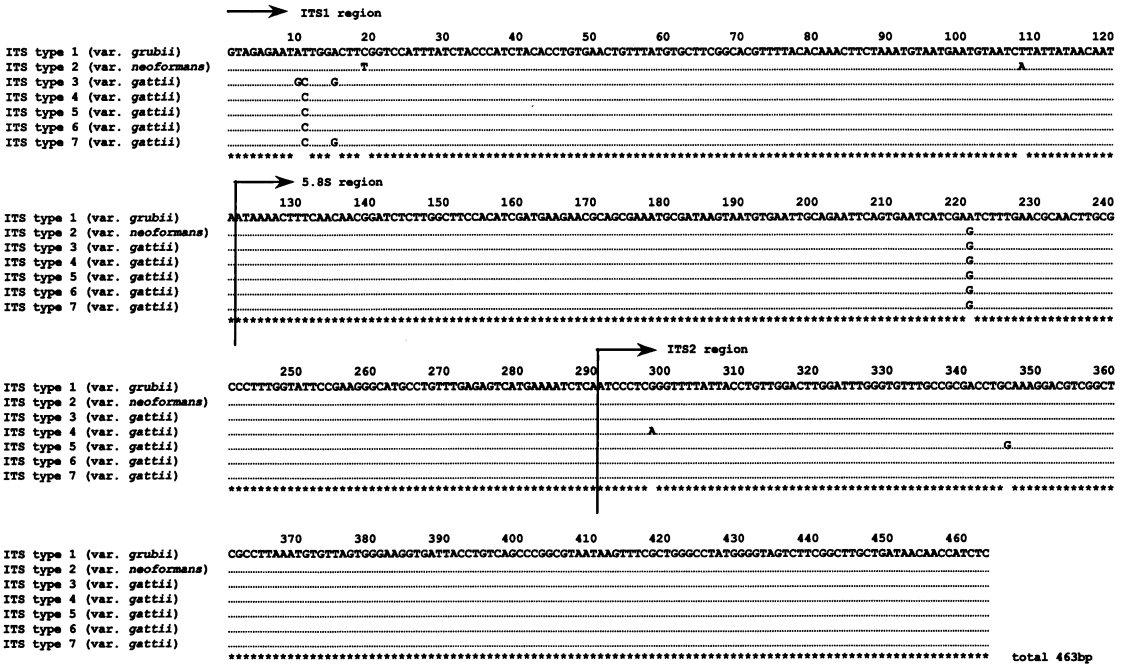

Fig. 1. Alignment of ITS sequences of all three varieties of *C. neoformans*, representing a sequence of each ITS type. Discriminatory positions 10, 11, 15, 19 and 108 in the ITS1 region, 221 in the 5.8S rRNA gene, and 298 and 346 in the ITS2 region are indicated.

for 11 *C.n.* var. *grubii* strains, seven *C.n.* var. *neoformans* strains, 10 serotype AD hybrid strains and 68 *C.n.* var. *gattii* strains. EMBL and DDBJ accession numbers for these sequences are given in Table 1. All *C. neoformans* strains revealed an identical ITS1-5.8S-ITS2 region length of 463 bp. The outgroup cryptococcal species investigated possessed different ITS region lengths. The ITS sequence of *C. luteolus* CBS943 was 432 bp (EMBL accession number AJ493576), *C. curvatus* CBS570 was 382 bp (EMBL accession AJ493577) and *C. laurentii* WM601 was 431 bp (EMBL accession AJ493578).

The sequences were aligned and searched for variety or molecular type-specific sequence signatures. All *C.n.* var. *grubii* (serotype A) strains revealed identical ITS sequences. The same was found for all *C.n.* var. *neoformans* (serotype D) strains. In addition the *C.n.* var. *neoformans* strains had identical sequences to the serotype AD hybrid strains investigated when the obtained PCR products were

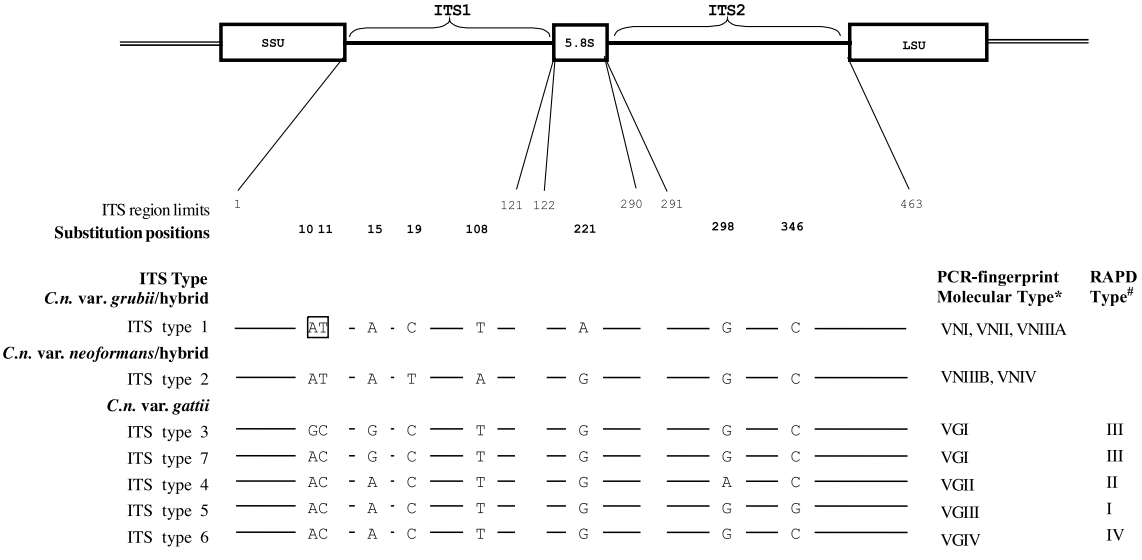

Fig. 2. ITS sequence signatures which make it possible to separate between *C.n.* var. *grubii*, *C.n.* var. *neoformans* and *C.n.* var. *gattii*, as well as between the molecular types of *C.n.* var. *gattii*, based upon eight combinations of variable nucleotides at positions 10, 11, 15, 19 and 108 in the ITS1 region, 221 in the 5.8S rDNA gene, and 298 and 346 in the ITS2. VNIIIA corresponds to the ITS type 1 allele sequence and VNIIIB corresponds to the ITS type 2 allele sequence obtained from the AD hybrid strains after cloning the amplified ITS1-5.8S-ITS2 region.

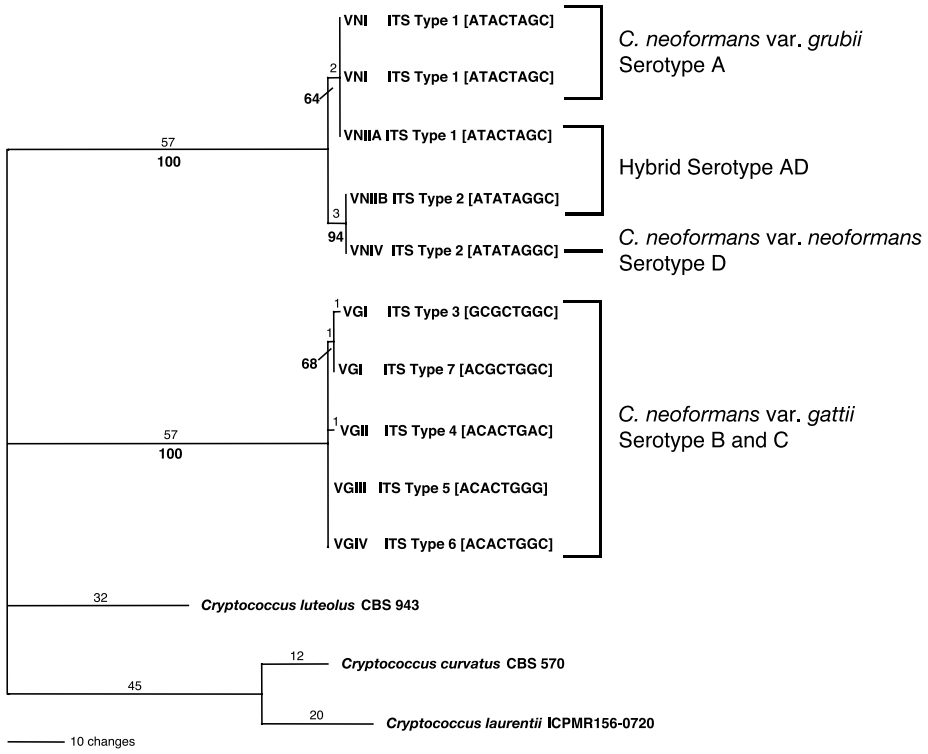

Fig. 3. Phylogenetic analysis of the seven ITS sequences observed among *C.n.* var. *grubii*, *C.n.* var. *neoformans* and *C.n.* var. *gattii*. The heuristic search (PAUP* 4.0b10) resulted in six most parsimonious trees of which one is presented. Numbers above the branches represent the branch length and numbers below the branches indicate the proportions above 50 of the 100 bootstrap replications in which a given branch appears. Branch lengths are proportional to the character state changes as indicated on the marker bar. The tree consists of 173 steps and reveals a CI of 0.9711, RI of 0.9609, and HI of 0.0289. VNIIIA represents the ITS type 1 allele sequence and VNIIIB the ITS type 2 allele sequence obtained from the AD hybrid (molecular type VNIII) strains after cloning the amplified ITS1-5.8S-ITS2 region.

sequenced direct. In contrast to the low variation found in the serotype A, D and AD strains, revealing only two ITS sequencing types, the 68 strains studied from *C.n.* var. *gattii* exhibited five different sequence types. The following ITS types were found within the *C. neoformans* complex: ITS type 1 = *C.n.* var. *grubii* (serotype A) and the serotype A allele of the AD hybrid; ITS type 2 = *C.n.* var. *neoformans* (serotype D), the serotype D allele of the AD hybrid and the serotype AD hybrid (if sequenced directly from the obtained PCR product); ITS type 3, ITS type 4, ITS type 5, ITS type 6 and ITS type 7 all correspond to *C.n.* var. *gattii* (serotypes B and C). Fig. 1 shows a sequence alignment including one representative sequence for each observed ITS type. The sequences representing *C.n.* var. *grubii* and *C.n.* var. *neoformans* were distinguishable from *C.n.* var. *gattii* by one nucleotide at position 11 of the ITS1 region (T in *C.n.* var. *grubii* and *C.n.* var. *neoformans* or C in *C.n.* var. *gattii*). *C.n.* var. *grubii* showed sequence variation at nucleotide positions 19 and 108 of the ITS1 region from those of *C.n.* var. *neoformans* (C or T in position 19 and T or A in position 108 in *C.n.* var. *grubii* or *C.n.* var. *neoformans* respectively). *C.n.* var. *grubii* showed a characteristic base A at nucleotide position 221 in the 5.8S rDNA gene where there was a G in the other two varieties. Based upon the sequence difference at positions 11, 19, 108 and 221 of the ITS1 and 5.8S rRNA gene, the three varieties were found to be delineated by the combination of these four bases:CCTG for *C.n.* var. *gattii*, TCTA for *C.n.* var. *grubii* and TTAG for *C.n.* var. *neoformans* (Figs. 1 and 2).

3.2. Cloning of serotype AD ITS regions

The ITS1-5.8S-ITS2 regions amplified from one serotype AD strain (IFM46138), representing the *URA5* hybrid between VNI and VNIV [15], were cloned and then randomly selected clones were individually re-sequenced, to determine whether the hybrid strains are haploid or diploid at the ITS locus. All cloned sequences were found to harbor either the ITS type 1 or the ITS type 2 allele sequence, corresponding to the serotype A or D ITS sequence, respectively. This confirmed that the serotype AD hybrid is diploid at the ITS1-5.8S-ITS2 locus.

3.3. ITS signature sequences for subtyping C.n. var. gattii

The ITS region including the 5.8S rRNA gene was sequenced for 68 strains of *C.n.* var. *gattii* and compared with those from *C.n.* var. *grubii*, *C.n.* var. *neoformans* and the AD hybrid. The ITS types were defined based upon the sequences at four variable positions, positions 10 and 15 in the ITS1 region, and 298 and 346 in the ITS2 region.

Of the 68 strains, 14 were classified as AAGG = ITS type 5, 29 as AAAC = ITS type 4, 16 as GGGC = ITS type 3, and seven as AGGC = ITS type 7 (Fig. 1). Two strains (IFM50896 and B-5742) could not be assigned to any of these groups; the four-base sequence combination for these strains was AAGC. It was observed that the AAGC ITS type has greatest similarity to, and possible origins within, the AAAC and AGGC types. Therefore the AAGC sequence type was designated a new ITS type 6 (Fig. 1). ITS sequencing divided all 68 studied *C.n.* var. *gattii* strains into five ITS types.

3.4. ITS sequence signatures to subtype all varieties and molecular types of C. neoformans

Based on the combination of eight different bases at the positions of 10, 11, 15, 19, 108 (ITS1 region), 221 (5.8S region), 298, and 346 (ITS2 region), we found that the 96 strains of *C. neoformans* could be classified into seven ITS types, which could be correlated to molecular types previously determined by PCR fingerprinting [12,14,16] or RAPD analysis [17]: ITS type 1 (ATACTAGC = *C.n.* var. *grubii*; molecular type VNI and VNII and the serotype A allele of the AD hybrid (VNIIIA), ITS type 2 (ATATAGGC = the serotype D allele of the AD hybrid (VNIIIB), AD hybrid after direct sequencing of the PCR product and *C.n.* var. *neoformans*; VNIV), ITS type 3 (GCGCTGGC) and ITS type 7 (ACGCTGGC) both = *C.n.* var. *gattii*; VGI = RAPD type III), ITS type 4 (ACACTGAC = *C.n.* var. *gattii*; VGII = RAPD type II), ITS type 5 (ACACTGGG = *C.n.* var. *gattii*; VGIII = RAPD type I), and the new ITS type 6 (ACACTGGC = *C.n.* var. *gattii*; VGIV) (Fig. 2).

3.5. Phylogenetic analysis of the ITS1-5.8S-ITS2 region

In order to determine the phylogenetic relatedness between the different molecular types and varieties of *C. neoformans*, representative sequences of each ITS type and previously identified molecular types were aligned and a phylogenetic analysis was carried out using the program PAUP* 4.0b10, generating six maximum-parsimony trees, each with a tree length of 173 steps, a consistency index (CI) of 0.9711, a retention index (RI) of 0.9609, and a homoplasy index (HI) of 0.0289. The consensus tree generated from this analysis clustered the obtained ITS sequences into two major branches with the first one comprising the ITS type 1 (*C.n.* var. *grubii* = molecular types VNI and VNII and the serotype A allele of the AD hybrid, VNIIIA) and ITS type 2 (serotype D allele of the AD hybrid (VNIIIB) and *C.n.* var. *neoformans*, VNIV) and the second cluster comprising all five *C.n.* var. *gattii* ITS types: ITS types 3 and 7 (both VGI = RAPD type III), ITS type 4 (VGII = RAPD type II), ITS type 5 (VGIII = RAPD type I) and the newly discovered ITS type 6 (VGIV) (see Fig. 3). The first cluster was subdivided into two branches, corresponding to ITS type 1 (*C.n.* var. *grubii* and the serotype A allele of the AD hybrid) or ITS type 2 (*C.n.* var. *neoformans* and the serotype D allele of the AD hybrid).

3.6. PCR fingerprinting and RAPD analyses

PCR fingerprinting with the minisatellite-specific primer M13 and the microsatellite-specific primer $(GACA)_4$ previously grouped over 1000 clinical and environmental isolates into eight major molecular types [12,14,16]. RAPD analysis has previously grouped isolates of *C.n.* var. *gattii* into three RAPD types. The PCR fingerprinting molecular type VGI (corresponding to the RAPD type III) was further subdivided by ITS sequence analysis into two ITS types: ITS type 3 and ITS type 7, which differed by one nucleotide at position 10 of the ITS1 region. Examples of PCR fingerprints obtained from ITS type 7 strains are shown in comparison to the eight molecular PCR fingerprinting standards in Fig. 4. No specific differences were found in the PCR fingerprinting profiles or RAPD patterns within the ITS type 7 isolates, when compared to those of ITS type 3.

The *C.n.* var. *gattii* strains IFM50896 and B-5742, previously assigned to molecular type VGIV by PCR fingerprinting, were observed to have a unique ITS1-5.8S-ITS2 sequence, and were assigned a new grouping, ITS type 6. This subgroup of *C. neoformans* strains had not been found in the RAPD analysis conducted previously in Ja-

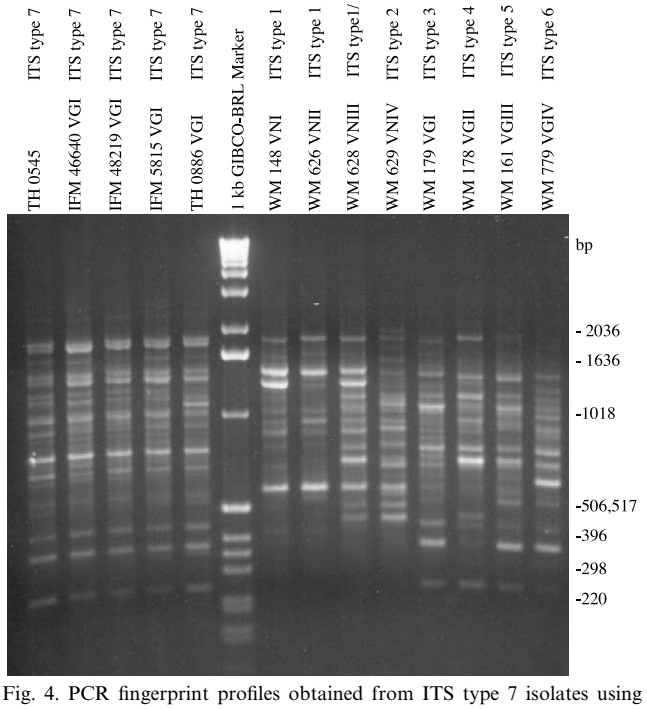

Fig. 4. PCR fingerprint profiles obtained from ITS type 7 isolates using the microsatellite-specific primer M13 (5′-GAG GGT GGC GGT TCT-3′) as a single primer in the PCR. Lanes 1–5 = ITS type 7 isolates, lane 6 = 1-kb Gibco BRL molecular marker, lanes 7–14 = the molecular type standard strains.

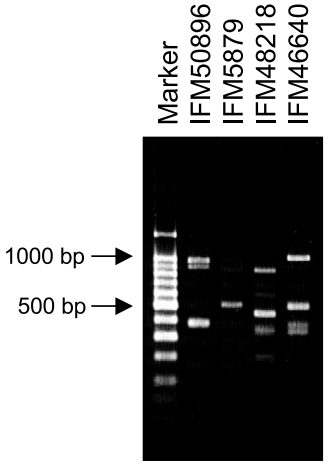

Fig. 5. RAPD patterns of the new genotype RAPD type IV of *C.n.* var. *gattii* of strain IFM50896 (ITS type 6) compared to representative strains of other ITS types: IFM5879 (ITS type 5 = RAPD type I), IFM48218 (ITS type 4 = RAPD type II), and IFM46640 (ITS type 7 = RAPD type III).

pan [17]. RAPD analysis was carried out on the ITS type 6 = VGIV strains in this study, and compared with the previously established RAPD patterns. The RAPD patterns of the ITS type 6 strains were different from representative strains of the other ITS types: IFM5879 (ITS type 5 = VGIII = RAPD type I), IFM48218 (ITS type 4 = VGII = RAPD type II), and IFM46640 (ITS type 7 = VGI = RAPD type III). RAPD bands of approximately 380, 890 and 980 bp were observed to be characteristic for the ITS type 6 strains, and were not present in the RAPD profiles of strains belonging to any other ITS types (Fig. 5). A new RAPD patterns, RAPD type IV, was established, which corresponds to the PCR fingerprinting molecular type VGIV.

4. Discussion

Variation within the ITS regions including the 5.8S rRNA gene for the three varieties of *C. neoformans* and the AD hybrid was examined via sequencing to measure intraspecific variation. In the current study, five variable nucleotide (nt) positions were observed within ITS1, one within the 5.8S rRNA gene, and two within the ITS2 region. Previous comparisons of the cryptococcal ITS regions observed only three variable positions within the combined ITS1 and ITS2 regions between isolates representing serotypes A, D, B, and C [24]. Given that only four strains were used in that study, certainly the variable nucleotide positions observed with different molecular types, particularly within *C.n.* var. *gattii*, could not be discerned. In addition, the ITS1, 5.8S, and ITS2 regions were reported to be 124 nt, 158 nt and 187 nt in length (total region length = 469 nt), respectively [24], whereas in the current study the ITS1 region was observed to be 121 nt, the 5.8S gene 169 nt, and the ITS2 region 173 nt in length (total region length = 463 nt). Close examination of the sequences of Fan et al. [24] available from the NCBI database (http://www.ncbi.nlm.nih.gov) revealed that the ITS1, 5.8S, and ITS2 regions were 120 nt, 169 nt, and 173 nt in length (total = 462 nt), respectively, despite the different lengths originally reported [24]. Alignment of their rDNA sequences (accession numbers L05428 and L14068) [24] to the ITS sequences in the herein reported study revealed a single deletion of a T nucleotide in the sequence obtained by Fan and co-workers, at position 76 of the alignment, corresponding to the ITS1. The sequences observed in the current study did not have any insertions or deletions causing length variability, and are in agreement with those sequences of the locus reported in previous studies [17]. The observed deletion within the sequence of Fan et al. [24], may be attributed to the relatively crude sequencing techniques available at that time.

In addition, the current analysis revealed that each variety of *C. neoformans* can be clearly differentiated based upon its ITS sequence. A single variable nucleotide was also found at position 19 of the ITS1 region, comprising a T in *C.n.* var. *neoformans* and the serotype D allele of the AD hybrid, and a C in the *C.n.* var. *gattii*, *C.n.* var. *grubii* and the serotype A allele of the AD hybrid. Comparison of the 5.8S gene sequences from 68 strains of *C.n.* var. *gattii*, seven *C.n.* var. *neoformans* strains and 10 serotype AD hybrid strains with those obtained from 11 *C.n.* var. *grubii* strains revealed a single nucleotide difference at position 221, with G for the first group and A for *C.n.* var. *grubii*. Hence, by determining the base composition at position 19 in the ITS1 region and position 221 of the 5.8S region, all three varieties could be differentiated with *C.n.* var. *grubii* CA, *C.n.* var. *neoformans* TG and *C.n.* var. *gattii* CG.

Nucleotide differences delineate *C.n.* var. *gattii* and *C.n.* var. *grubii* (including the serotype A allele of the AD hybrid) from *C.n.* var. *neoformans* (including the serotype D allele of the AD hybrid) at the ITS1 locus. From these results, we propose that the four nucleotide positions 11, 19, 108 and 221 should form the basis for the differentiation of the three varieties, having nucleotide combinations of TCTA = *C.n.* var. *grubii*, TTAG = *C.n.* var. *neoformans*, and CCTG = *C.n.* var. *gattii*. This single PCR step/sequencing method is simple and reliable due to the small amplified ITS band size of 463 bp.

It was observed that cloned ITS sequences from serotype AD revealed two ITS sequence types: ITS type 1 = serotype A allele and ITS type 2 = serotype D allele, indicating that the serotype AD is diploid at this locus. These results confirmed the results found in a previous study examining the ploidy of the serotype AD [22]. This study observed that strains of serotype AD comprised either two or three different copies of the *URA5* gene, which corresponded to *URA5* sequences from strains corresponding either to molecular types VNI, VNII and VNIV or VNII and VNIV, suggesting different recombination events be-

tween *C.n.* var. *grubii* and *C.n.* var. *neoformans* leading to diploid or triploid strains [16,22]. If cloned ITS sequences from serotype A, B or C strains were sequenced, only a single ITS type per sequenced strain was obtained, confirming the haploid nature of each of these strains. No heterogeneity in the sequences of this locus of the rRNA repeat unit was found within a single haploid serotype A, B or C strain (data not shown). For unknown reasons, direct sequencing of the ITS1-5.8S-ITS2 locus from the AD hybrid strains, without prior cloning, revealed always only the ITS type 2. The two alleles of this locus of the AD hybrid strains could only be obtained after cloning of the PCR product. A similar phenomenon was observed when sequencing other parts of the rRNA locus (data not shown) or the *URA5* gene [22].

Interestingly, it was found that *C.n.* var. *gattii* strains belonging to molecular type VGI and RAPD type III could be further sub-typed based upon their ITS genotype. Strains in this group were observed to belong to either ITS type 3 or ITS type 7. Isolates from Australia and Papua New Guinea were observed to belong to ITS type 7 (Table 1), and most of the strains of ITS type 3 originated in Asian countries. Further investigations into the geographical associations of these ITS types would be of interest.

Clear associations were observed between the ITS types observed herein, and PCR fingerprinting and RAPD profiles observed in previous studies. In addition, by correlation of known PCR fingerprinting, RAPD and amplified fragment length polymorphism types [10], it has been possible to infer the relationship of ITS types to the genotypes determined in a study of the intergenic spacer (IGS) of *C. neoformans* [25]. ITS type 1 corresponds to the IGS genotypes 1A, 1B, and 1C, ITS type 2 corresponds to IGS genotypes 2A, 2B, and 2C. The ITS types 3 and 7 correspond to IGS genotype 4, ITS type 4 corresponds to IGS genotype 3, and ITS type 5 to IGS genotype 5. Representatives of ITS type 6 (PCR fingerprint molecular type VGIV, RAPD type IV) were not included in the published IGS study. However, the currently estimated phylogenetic relationships between isolates of each of the seven observed ITS types are in concordance with those determined using the IGS region [25].

C.n. var. *gattii* was first reported to be associated with *Eucalyptus* trees and the dispersal of this agent has been proposed to be related to eucalypt exportation from Australia throughout the world [7,12,26]. It has been found that clinical and environmental isolates of *C.n.* var. *gattii* from Australia and the USA could be classified into three major profiles (VGI, VGII and VGIII) using the RAPD method and PCR fingerprinting [7,13]. Among the three profiles, a predominance of type VGI was reported [7,13]. Our analysis of *C.n.* var. *gattii* showed that the clinical and environmental strains with the VGI molecular type from the USA [12,14,16] and RAPD type III profile [17] belong to ITS type 3, and other strains with the VGIII molecular type [12,14,16] = RAPD type I [17] belonged to ITS type 5. These data support previous suggestions of the possibility of a recent fungal transfer due to eucalypt exportation from Australia to the USA [7,26].

Of the 68 *C.n.* var. *gattii* strains, two (IFM50896 and B-5742) revealed a novel ITS genotype. These isolates, originating from Johannesburg, South Africa, and Punjab, India, respectively, both belong to the PCR fingerprinting molecular type VGIV [12,15]. VGIV has been isolated infrequently only among isolates from South Africa, India, and some countries within South America [16,27]. Our data support these PCR fingerprinting results, and we propose the designation of the new ITS type 6 for these strains. Since both strains were also found to belong to serotype C, the ITS genotypes among the other serotype C strains in the study were investigated. ITS typing results from the four other serotype C strains in the study (CBS6993, IFM5873, IFM5875, and IFM5883) revealed that they belonged to ITS type 5, corresponding to molecular type VGIII = RAPD type I. These data suggest that the serotype does not necessarily conform to ITS genotype delineations within *C.n.* var. *gattii*. Similar observations were made in molecular epidemiology studies using PCR fingerprinting [12,14,16,28], which suggests that serotyping is not a reliable typing method for *C. neoformans*.

From the point of view of DNA sequence similarity, ITS type 6 (ACACTGGC) (VGIV = RAPD type IV) has greater similarity to ITS type 5 (ACACTGGG) (VGIII = RAPD type I) and ITS type 4 (ACACTGAC) (VGII = RAPD type II) than to ITS type 3 (GCGCTGGC) and ITS type 7 (ACGCTGGC) (both VGI = RAPD type III), based upon the rate of nucleotide substitutions. This level of heterogeneity was not observed among *C.n.* var. *neoformans* and *C.n.* var. *grubii* ITS sequences. Xu et al. [29], in a study of several cryptococcal genes, including the ITS1-5.8S-ITS2, estimated the divergence of *C.n.* var. *gattii* from *C.n.* var. *grubii* and *C.n.* var. *neoformans* to be 37 million years ago, whereas the divergence of *C.n.* var. *grubii* and *C.n.* var. *neoformans* was estimated at 18.5 million years ago. The divergence of serotypes B and C was estimated at 9.5 million years ago. Our observations of significantly greater diversity among *C.n.* var. *gattii* compared to that observed in *C.n.* var. *grubii* or *C.n.* var. *neoformans* support these estimates. *C.n.* var. *grubii* has been recently established [6] and the findings of this study support a phylogenetically fixed position of *C.n.* var. *grubii*, being distinct from the other two varieties of *C. neoformans*, but more closely related to *C.n.* var. *neoformans* than to *C.n.* var. *gattii* (see Fig. 3).

This study revealed further evidence for the delineation of three varieties within the *C. neoformans* species complex by providing a fast and informative technique to identify *C. neoformans* isolates in clinical laboratories to the variety level. It also indicates evidence for ongoing speciation within the *C. neoformans* species complex, by further subtyping of *C.n.* var. *gattii* isolates to the molecular type

level and demonstrating the origin of the two alleles of the ITS1-5.8S-ITS2 locus of the AD hybrid strains.

Acknowledgements

This work was supported by the Frontier Studies and International Networking of Genetic Resources in Pathogenic Fungi and Actinomycetes (FNGRPF) program, through the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, of the Japanese Government in 2001 to Y.M. and the National Health and Medical Research Council of Australia, NH and MRC Grants 960792 and 990738 to W.M. S.K. and W.M. would like to thank Matthew Huynh for his help with the ITS cloning and sequencing.

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