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# Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*

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## Abstract

This communication describes the consensus multi-locus typing scheme established by the Cryptococcal Working Group I (Genotyping of *Cryptococcus neoformans* and *C. gattii*) of the International Society for Human and Animal Mycology (ISHAM) using seven unlinked genetic loci for global strain genotyping. These genetic loci include the housekeeping genes *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5* and the IGS1 region. Allele and sequence type information are accessible at http://www.mlst.net/.

### Keywords

*Cryptococcus neoformans*; *Cryptococcus gattii*; Genotyping; Multi Locus Sequence Typing

## Introduction

*Cryptococcus neoformans*, the agent of cryptococcosis, had been considered a homogeneous species until 1949 when the existence of four serotypes was revealed based on the antigenic properties of its polysaccharide capsule [1]. Such heterogeneity of the species, however, remained obscure until the two morphologically distinct teleomorphs of *C. neoformans* were discovered during the mid 1970s [2,3]. The teleomorph *Filobasidiella neoformans* was found



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to be produced by strains of serotype A and D [2] while *F. bacillispora* was found to be produced by strains of serotype B and C [3]. Ensuing studies revealed numerous differences between the anamorphs of the two *Filobasidiella* species with regards to their ecology, epidemiology, pathobiology, biochemistry and genetics.

Presently, the etiologic agent of cryptococcosis is classified into two species [4], *C. neoformans*, with two varieties: *C. neoformans* var. *grubii* (serotype A) [5] and *C. neoformans* var. *neoformans* (serotype D) [6], as well as an AD hybrid, and *C. gattii* (serotypes B and C) [7]. Intra-species genetic diversity has also been revealed as more genotyping methods have been applied for each serotype. In addition inter-species hybrid strains of AB and BD serotypes have been described [8,9]. As a result, the number of scientifically valid species within *C. neoformans* has become a controversial issue because of the differing opinions among taxonomists as to the appropriate definition of a species. There are several research groups focusing on the molecular determination of the number of genetically diverse sub-groups within each serotype. The molecular methods employed by each group to define these sub-groups vary from DNA fingerprinting [10,11] and PCR fingerprinting based on microsatellite-(M13) or minisatellite-specific primers (e.g., $(GACA)_4$ or $(GTG)_5$) [12–16], over random amplification of polymorphic DNA (RAPD) analysis [17–20], amplified fragment length polymorphism (AFLP) analysis [21–23], restriction fragment length polymorphism (RFLP) analysis of the *URA5* [16,24] and *PLB1* genes [25], the use of IGS sequences [26], multigene sequence analysis [27, Meyer *et al.* unpublished data], to multi-locus sequence typing (MLST) [23,28] and multi-locus microsatellite typing (MLMT) [29,30]. This research has revealed associations between geographic origin and particular genotypes, implying an epidemiologic significance of certain genotypes. Different methods have resulted in various numbers of sub-groups or different nomenclature of those sub-groups. However, due to the lack of a cross-reference consensus between the results obtained by different genotyping method, there is currently no concordance on a universally acceptable genotyping method for this important human pathogen.

Recognizing the urgent need for a standardized globally acceptable typing method, a Cryptococcus working group I, 'Genotyping of *Cryptococcus neoformans* and *C. gattii*', was formed under the umbrella of the International Society of Human and Animal Mycoses (ISHAM) in the beginning of 2007 which united all the major research groups that were involved in molecular strain typing of *C. neoformans* complex. The members of this ISHAM working group met at the 3rd Trends in Medical Mycology (TIMM3) Meeting in Torino, Italy in October 2007, and reviewed all the typing techniques in use. The group selected multi-locus sequence typing (MLST) as the method of choice for future strain typing in light of its high discriminatory power as well as reproducibility between different laboratories. The working group also chose standard reference strains representing the eight known major molecular types of the agent of cryptococcosis as well as the nomenclature of each genotype.

## Consensus genotype nomenclature

As a result of the Torino meeting, the working group recognized that the different genotyping methods used by the different research groups lead to corresponding major genotypes for the agents of cryptococcosis (Table 1). Principally, the two main typing systems being used are: PCR fingerprinting using primers specific for microsatellite (M13) [14,16] or minisatellite $(GACA)_4$ DNA [13,15] and AFLP analysis [21]. In both typing schemes, over 2000 isolates were grouped into eight major molecular types. With some exceptions [26,31], the molecular types of *C. neoformans* are correlated with the serotypes: *C. neoformans* var. *grubii*, serotype A, consists of molecular types VNI=AFLP1 and VNII=AFLP1A; the hybrid serotype AD comprises VNIII=AFLP3; and *C. neoformans* var. *neoformans*, serotype D, corresponds to VNIV=AFLP2. *C. gattii* consists of VGI=AFLP4, VGII = AFLP6, VGIII=AFLP5, and

VGIV=AFLP7, which all correspond to both serotypes B or C [16,21, unpublished data]. Based on these findings, it was agreed by all cryptococcal working group members present in Torino to use the VNI–VNIV and VGI–VGIV nomenclature [16] since it correlated with the current concept of two species and represents the global population structure based on more than 2000 *C. neoformans* and *C. gattii* isolates among which *C. neoformans* var. *grubii* (serotype A=VNI) being the most prevalent molecular type world-wide.

## Consensus standard strains

To enable global standardization, the working group also agreed to use a set of standard strains representing each of the eight major molecular types. This included the molecular type strains used in PCR fingerprinting or *URA5*-RFLP analysis [16] plus additional strains representing type cultures or strains, which are used in major cryptococcal genome projects (Table 2). All standard strains are publicly available from the CBS-Fungal Biodiversity Centre (CBS) (http://www.cbs.knaw.nl), the American Type Culture Collection (ATCC) (http://www.atcc.org) or the Fungal Genetic Stock Center (FGS) (http://www.fgsc.net). The corresponding collection numbers are listed in Table 2.

## Consensus multi-locus sequence typing loci

To overcome problems arising from inter-laboratory reproducibility associated with the two commonly used typing techniques, such as PCR fingerprinting or AFLP analysis, the working group decided to use multi-locus sequence typing (MLST) as the method of choice for future cryptococcal strain typing. MLST has become the number one typing approach for epidemiological investigations of microorganisms [32]. MLST, originally developed for bacteria [32], indexes the sequence variation in approximately 400–500 bp of five to ten genes composed primarily of housekeeping genes. This technique has proven to be highly discriminatory for a number of human pathogenic fungi: *C. albicans* [33], *C. glabrata* [34], *C. tropicalis* [35], *Coccidioides* spp. [36] and *Histoplasma capsulatum* [37]. Most of the published MLST schemes are developed as tools for the wider scientific community, by being made publicly available as online databases at http://www.mlst.net/ and http://pubmlst.org/. In the case of the *Cryptococcus* species complex, two different MLST typing schemes have been introduced to type isolates of *C. neoformans* [23], and *C. gattii* [28], using twelve and eight unlinked loci respectively.

In the first study, 12 unlinked polymorphic loci: *MPD1*, *TOP1*, *MP88*, *CAP59*, *URE1*, *PLB1*, *CAP10*, *GPD1*, *TEF1*, *SOD1*, *LAC1* and the IGS1 ribosomal RNA intergenic spacer region, which are dispersed on nine different chromosomes, were used to type 102 globally obtained serotype A strains [23]. MLST differentiated three major groups among the studied isolates, corresponding to VNI, VNII and VNB, a Botswana specific genotype closely related to VNI. In connection with this study a central web based database was created at www.mlst.net (http://cneoformans.mlst.net/) allowing for an online determination of the alleles and sequence types of *C. neoformans* serotype A strains.

The second study used eight unlinked polymorphic loci: *SXIa* or *SXIα*, IGS1, *TEF1*, *GPD1*, *LAC1*, *CAP10*, *PLB1*, and *MPD1*, of which two are mating type locus specific and can not be amplified for all strains, to type 202 *C. gattii* strains. These loci were supplemented for a more detailed analysis of 9 closely related strains by 22 additional gene loci: *HOG1*, *BWC1*, *CNB1*, *TOR1*, *CAC1*, *CRG1*, *URE1*, *FHB1*, *BWC2*, *CNA1*, *CBP1*, *TSA1*, *STE7*, *FTR1*, *PAK1*, *CAP59*, *ICL1*, *GPA1*, *GPB1*, *RAS1*, *CCP1*, and *TRR1* to investigate the origin of the Vancouver Island outbreak isolates [28]. MLST differentiated all four major molecular types of *C. gattii* (VGI, VGII, VGIII and VGIV) and highlighted two possible origins (Australia or South America) for the outbreak strains.

Statistical analysis using the Simpsons's index of diversity [38] revealed that for both previously studied MLST data sets, a minimum of seven loci are required to differentiate between the sequence types of all strains (Fig. 1). For the Litvintseva *et al.* [23] MLST data set, the following loci resulted in the highest discrimination of the investigated strains: *CAP59*, IGS1, *GPD1, LAC1, PLB1, MP88* and *SOD1*, with a Simpson's index of diversity of 0.9632. For the Fraser *et al.* [28] MLST data set, the most discriminatory loci were: *GPD1*, IGS1, *TEF1, LAC1, MPD1, CAP10* and *PLB1*, which resulted in a Simpson's index of diversity of 0.9319.

Both MLST schemes utilized highly polymorphic loci, which resulted in stable and reproducible typing systems that were able to distinguish between closely related strains. While using as many genetic loci as possible would enhance the discriminatory power of the MLST scheme, it would be pragmatic to achieve the maximal level of differentiation with a minimal set of genetic loci. The ideal MLST scheme for the *Cryptococcus* species complex should fulfill two criteria: (i) it should amplify and type the same genes from all five serotypes/eight molecular types using the same set of primers, and (ii) the selected genes should contain sufficient sequence diversity to produce a discriminatory typing scheme. Taking these facts into account, the working group has selected a set of seven gene loci for a cryptococcal consensus MLST scheme based on the results obtained in the previously published studies by Litvintseva *et al.* [23], Fraser *et al.* [28], and additional unpublished data obtained by Meyer *et al.* and Fisher *et al.* Special emphasis was placed on using loci that exhibited the largest number of different allele types, as well as the potential to use the same primers with all eight major molecular types identified previously for *C. neoformans* and *C. gattii.* These gene loci included six housekeeping genes *CAP59, GPD1, LAC1, PLB1, SOD1, URA5*, from which three genes code for cryptococcal virulence factors: the polysaccharide capsule (*CAP59*), melanin synthesis (*LAC1*) and cell invasion (*PLB1*), and the intergenic spacer, IGS1, which was selected based on its high allelic diversity.

All the herein proposed MLST loci, except for the *CAP59* locus, are similar to the ones used previously enabling the incorporation of, and comparisons with all previously obtained data. The region of the *CAP59* locus proposed for the consensus MLST scheme represents a different fragment of the *CAP59* gene used by Litvintseva *et al.* [23] (Fig. 2). This new locus was chosen based on the fact that it can be amplified from all eight molecular types using the same primers.

An additional locus, *TEF1*, which also showed high discriminatory power when used for *C. neoformans* var. *grubii* and for *C. gattii* molecular type VGII, was excluded from the consensus typing scheme. This was based on the fact that sequence data are only available for *C. neoformans* var. *grubii* and technical problems had been encountered when amplifying this locus. However, this locus may offer additional discrimination in some of the eight major molecular types.

To enable amplification of all seven loci from the eight major molecular types of *C. neoformans* and *C. gattii*, the previously published primers were tested on all eight major molecular types in three of the six laboratories (Teun Boekhout's laboratory at the CBS, June Kwon Chung's laboratory at the NIH, Matthew Fisher's laboratory at the Imperial College, Wieland Meyer's laboratory at the University of Sydney, Tom Mitchell's laboratory at Duke University, and Maria Anna Viviani's laboratory at the Università degli Studi di Milano) that collaborated in the development of the herein presented consensus MLST scheme. Satisfactory amplifications were obtained for all loci except for the *SOD1* locus, where two different sets of primers were finally used to amplify either VNI–VNIV for *C. neoformans* or VGI–VGIV for *C. gattii* (Table 3). The specific primers and the suggested amplification conditions to amplify the seven gene loci are given in Table 3. Primer directions are listed according to the orientation in the genome sequence of the strain H99 at the Broad Institute

(http://www.broad.mit.edu). Variations in the quality of the amplification products, resulting from either the Taq DNA polymerase enzyme or the PCR machine and PCR conditions used, were observed between participating laboratories. For that reason, the amplification conditions given in Table 3 should only serve as a guideline that may be optimized by individual laboratories.

## Automatic allele type and sequence type retrieval

Allele types for *C. neoformans* were assigned according to Litvintseva *et al.* [23] and for *C. gattii* according to by Fraser *et al.* [28], if applicable. The exact start- and endpoints for the sequence of each analyzed locus are given in Table 3 based on the H99 genome sequence at the Broad Institute (http://www.broad.mit.edu/), these may change over time if more strains are studied. The latest sequence cut points are listed at the webpage for each locus. To standardize the assignment of allele types (AT) and sequence types (ST), a centralized globally accessible MLST database will be established at www.mlst.net/. The online software NRDB (http://linux.mlst.net/nrdb/nrdb.htm) allows for an automatic retrieval of allele and sequence types and will assign a new allele and sequence type for any submitted unknown sequence. These are then uploaded to the database *via* a database curator. The designated curators are contactable *via* the website.

## Conclusion

In conclusion the ISHAM working group on 'Genotyping of *Cryptococcus neoformans* and *C. gattii'* proposes the following set of genetic loci as an international standard for multi-locus sequence typing for *C. neoformans* and *C. gattii: CAP59, GPD1, LAC1, PLB1, SOD1, URA5* and IGS1.

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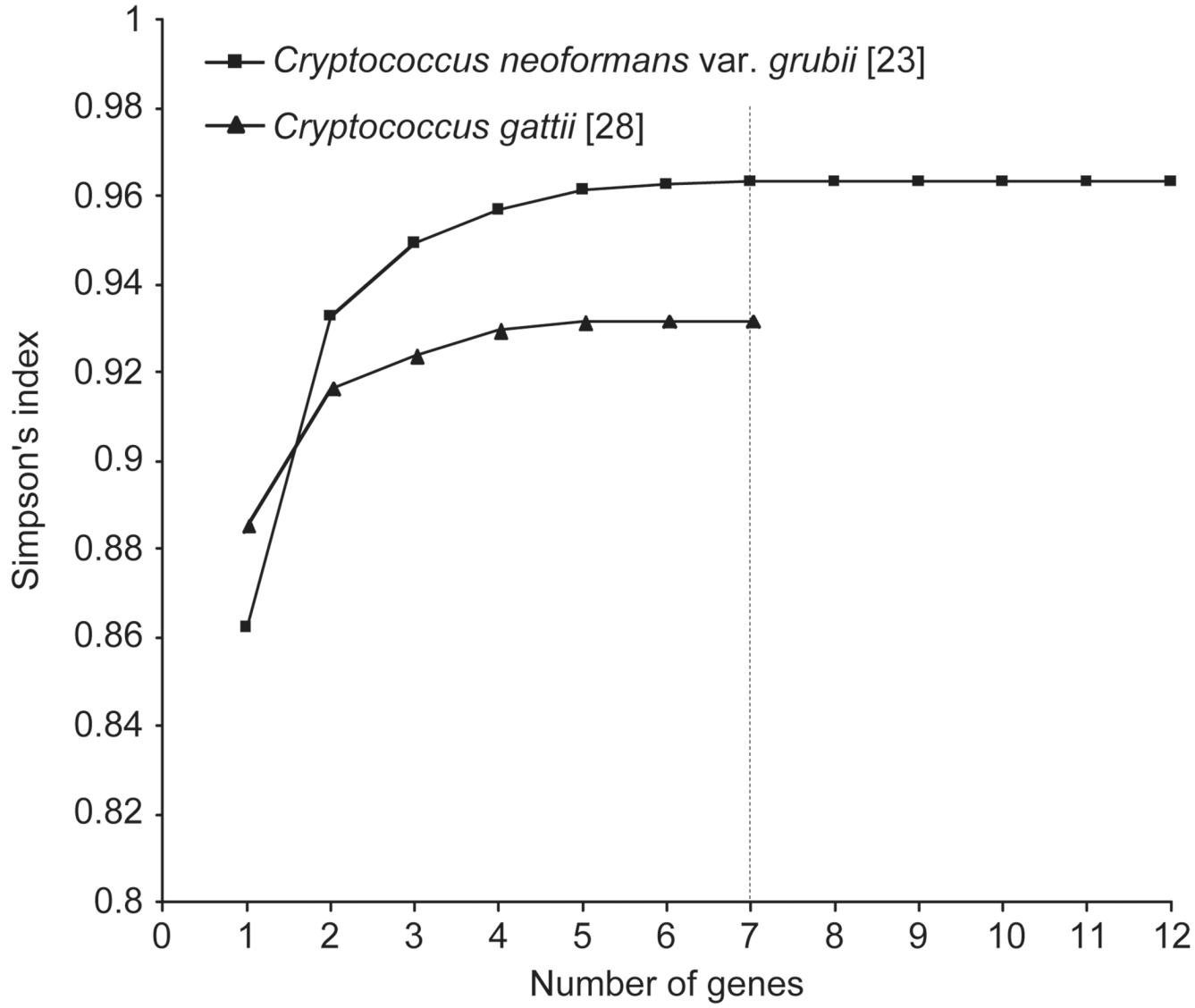


**Fig. 1.**

Number of genes necessary to differentiate all sequence types obtained in the two previously published cryptococcal MLST schemes based on Simpson's index of diversity [38]. For the Fraser *et al.* [28] data set the mating type locus specific genes, *SXI***a** or *SXIα*, have been excluded for this analysis since they can't be amplified from all isolates.

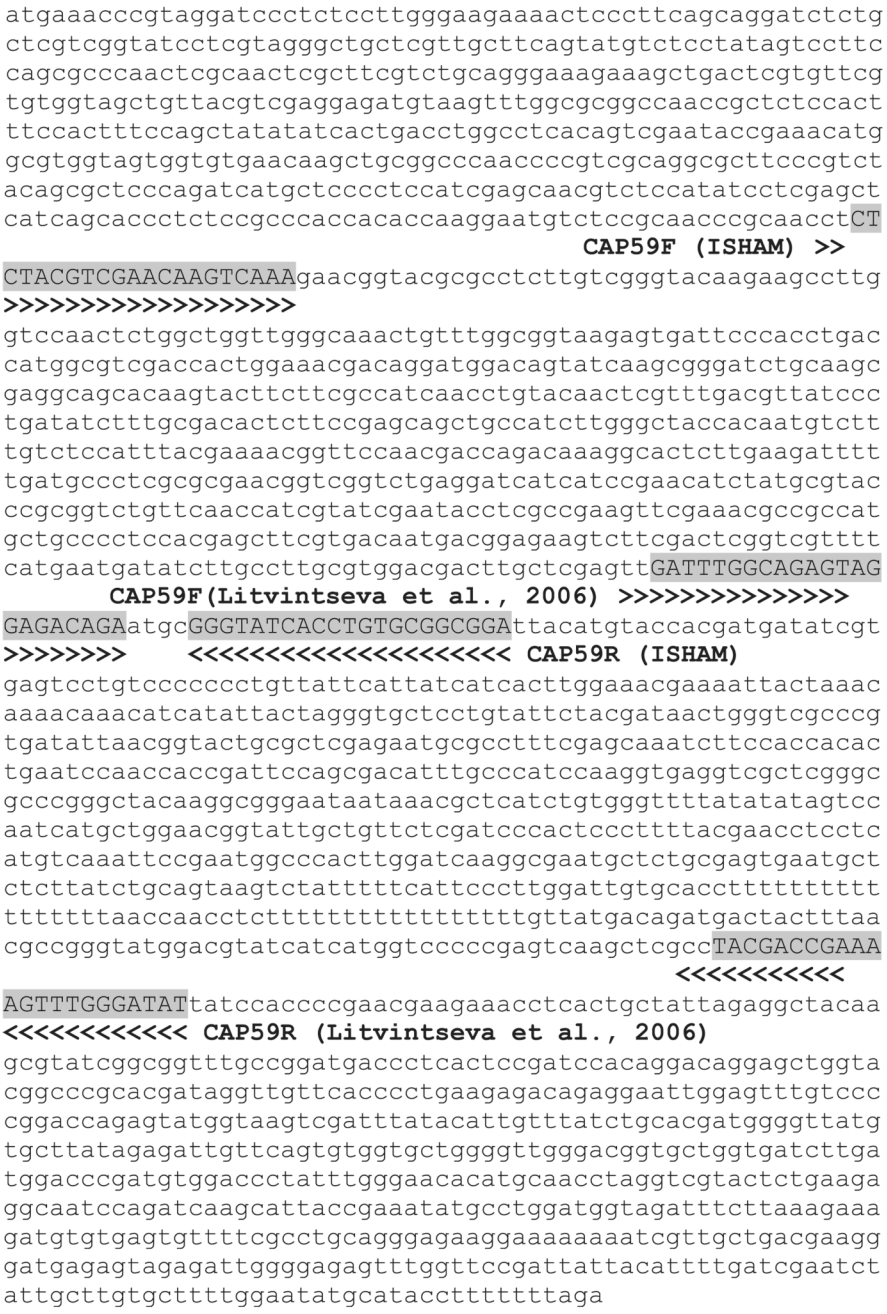


**Fig. 2.**

*CAP59* genomic sequence of strain H99 (*Cryptococcus neoformans* var. *grubii*, VNI, http://www.broad.mit.edu) used as master sequence, indicating the location of the Cryptococcal Working Group (ISHAM) proposed consensus primers used in Fraser *et al.* [28] and the location of the primers used in Litvintseva *et al.* [23].

**Table 1**

Concordance of different molecular typing methods used for *Cryptococcus neoformans* and *Cryptococcus gattii*

| Species/Variety/ Hybrid | Serotype | PCR-fingerprinting molecular type Meyer *et al*. [14,16,39] | PCR-fingerprinting molecular type Viviani *et al.* [13] | AFLP genotype Boekhout *et al*. [21] | AFLP genotype Litvintseva *et al*. [23] | *URA5* RFLP type Meyer *et al*. [16] | *PLB1* RFLP type Latouche *et al.* [25] | IGS genotype Diaz *et al*. [26,31] | ITS genotype Katsu *et al.* [40] |
|---|---|---|---|---|---|---|---|---|---|
| *C. neoformans var. grubii* | A | VNI | VN6 (VN5) | AFLP1 | VNI | VNI | A1 | 1A/1B | ITS1 |
| | A | VNII | | AFLP1A/AFLP1B | VNB | VNII | | 1A | ITS1 |
| | A | VNII | VN7 | AFLP1A/AFLP1B | VNII | VNII | A2 | 1C | ITS1 |
| AD Hybrid | AD | VNIII | VN3/VN4 | AFLP3 | | VNIII | A3 | 2C | ITS1/ITS2 |
| *C. neoformans var. neoformans* | D | VNIV | VN1 (VN2) | AFLP2 | | VNIV | A4 | 2A/2B /2C | ITS2 |
| *C. gattii* | B/C | VGI | | AFLP4A/AFLP4B | | VGI | A5 | 4 | ITS3/ITS7 |
| | B/C | VGII | | AFLP6 | | VGII | A6 | 3 | ITS4 |
| | B/C | VGIII | | AFLP5A/AFLP5B/ AFLP5C | | VGIII | A7 | 5 | ITS5 |
| | B/C | VGIV | | AFLP7 | | VGIV | A8 | 6 | ITS6 |

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**Table 2**

Standard/reference strains for *Cryptococcus neoformans* and *Cryptococcus gattii* strain typing

| CBS # | ATCC# | FGS# | Other numbers | MAT & Serotype | Comments | References |
|---|---|---|---|---|---|---|
| *Cryptococcus neoformans* | | | | | | |
| *Cryptococcus neoformans var. grubii* | | | | | | |
| VNI (Meyer *et al*. [14,16])=AFLP1 (Boekhout *et al.* [21])=VN6 (VN5) (Viviani *et al.* [13]) | | | | | | |
| CBS 10085 | ATCC MYA-4564 | 10415 | WM 148; W10; Brown | αA | 1989, Australia, NSW, Sydney, clinical, CSF, HIV–, isolated by Sharon Chen | [14,18] |
| CBS 8710 | ATCC 48922 | 9487 | DUMC 135.97; H99; NYSD 1649; CBS 10515; WM 04.15 | αA | 1978, USA, NC, Durham, clinical, CSF, patient with Hodgkin's lymphoma, isolated by John Perfect/Wiley Schell, type culture of *C. neoformans* var. *grubii* , genome sequence strain | [5] |
| VNII (Meyer *et al.* [14/16])=AFLP1A (Boekhout *et al*. [21])=VN7 (Viviani *et al*. [13]) | | | | | | |
| CBS 10084 | ATCC MYA-4565 | 10416 | WM 626, W20; Cetin | αA | 1993, Australia, NSW, Sydney, clinical, CSF, HIV–, isolated by Sharon Chen | [14,18] |
| AD hybrid | | | | | | |
| VNIII (Meyer *et al*. [14,16])=AFLP3 (Boekhout *et al*. [21])=VN33VN4 (Viviani *et al.* [13]) | | | | | | |
| CBS 10080 | ATCC MYA-4566 | 10417 | WM 628; 88B5400; Zapf | αA/*a*D | 1988, Australia, VIC, Melbourne, clinical, CSF, HIV+, isolated by Bryan Speed | [14,18] |
| CBS 132 | ATCC 32045 | - | CCRC 20528; DBVPG 6010; IFO 0608; IGC 3957; NRRL Y-2534 | αA/*a*D | 1894, Italy, environmental, fermenting fruit juice, isolated by F. Sanfelice, type culture for *C. neoformans* | [41] |
| *Cryptococcus neoformans var. neoformans* | | | | | | |
| VNIV (Meyer *et al.* [14,16])=AFLP2 (Boekhout *et al*. [21])=VN1 (VN2) (Viviani *et al*. [13]) | | | | | | |
| CBS 10079 | ATCC MYA-4567 | 10418 | WM 629; B 87455, Borg, F 14 | αD | 1987, Australia, VIC, Melbourne, clinical, blood, HIV+, isolated by Bryan Speed | [14] |
| CBS 6900 | ATCC 34873 | 10423 | B-3501; DBVPG 6228; CBS 7697 | αD | 1975, USA, MD, Bethesda, NIH, crossing of NIH 12 x NIH 433, isolated by June Kwon-Chung | [42] |
| *Cryptococcus gattii* | | | | | | |
| VGI (Meyer *et al*. [16])=AFLP4 (Boekhout *et al.* [21]) | | | | | | |
| CBS 10078 | ATCC MYA-4560 | 10419 | WM 179; Bryon; H33.1; MH56 | αB | 1993, Australia, NSW, Sydney, clinical, CSF, HIV −, isolated by Sharon Chen | [16,18] |
| CBS 6289 | ATCC 32269 | - | MUCL 30449, RV 20186; CBS 8273 | αB | 1966, Congo, Kinshasa, clinical, CSF, isolated by E. Gatti/R. Eeckels, type strain of *C. neoformans* var. *gattii*, | [43] |
| CBS 10510 | - | - | WM 276; TCS -SC1 | αB | 1993, Australia, NSW, Mt Annan National Park, environmental, *Eycalyptus tereticornis* woody debris, isolated by Tania Sorrell/Sharon Chen, genome sequence strain | [16] |

| CBS # | ATCC# | FGS# | Other numbers | MAT & Serotype | Comments | References |
|---|---|---|---|---|---|---|
| VGII (Meyer *et al.* [16])=AFLP6 (Boekhout *et al*. [21]) | | | | | | |
| CBS 10082 | ATCC MYA-4561 | 10420 | WM 178; 49435; Colter; IFM 50894 | αB | 1991, Australia, NSW, Sydney, clinical, CSF, HIV −, isolated by Sharon Chen | [16] |
| CBS 10514 | - | - | CDC R265; WM 02.32 | αB | 2001, Canada, BC, Duncan, Vancouver Island, clinical, bronchial wash, isolated by British Columbia CDC, high virulent Vancouver Island outbreak strain, VGIIa, genome sequence strain | [44] |
| VGIII (Meyer *et al.* [16])=AFLP5 (Boekhout *et al*. [21]) | | | | | | |
| CBS 10081 | ATCC MYA-4562 | 10421 | WM 175; WM 161; E698; 689; TP 0689; D1.13H | αB | 1992, USA, California, San Diego, Blind Recreation Center/Park Boulevard UPAS street, environmental, Eucalyptus spp. woody debris, isolated by Tania Pfeifer/David Ellis | [16,19] |
| CBS 6955 | ATCC 32608 | 10424 | DBVPG 6225; MUCL 30454; NIH 191; CBS 6916 | αC | Before 1970, USA, San Fernando, California, clinical, CSF. | [45] |
| VGIV (Meyer *et al.* [16])=AFLP7 (Boekhout *et al*. [21]) | | | | | | |
| CBS 10101 | ATCC MYA-4563 | 10422 | WM 779; King Cheetah; IFM 50896 | αC | 1994, South Africa, Johannesburg, veterinary, Cheetah, isolated by Valarie Davis | [16,46] |

**Table 3**

MLST loci information

| Gene locus | Gene product | Chromo-some location[a] | Primer name and sequence (If not specified differently primers listed will work for *C.n.* and *C.g.*) | Amplification conditions | No. of bases analysed (bp)[a] | Analysed sequence fragment, start (5′) and end (3′) points[a] | Ref. |
|---|---|---|---|---|---|---|---|
| *CAP59* | Capsular associated protein | 1 | **CAP59F** 5′ CTCTACGTCGA GCAAGTCAAG 3′<br>**CAP59R** 5′ TCCGCTGCA CAAGTGATACCC 3′ | 94°C 3min; 35 cycles: 94°C 30s, 568C 30s, 72°C 1min<br>**Alternative conditions:**<br>30 cycles: 94°C 30s, 64°C 30s, 72°C 1min<br>**or:**<br>30 cycles: 95°C 3min, 95°C 30s, 54°C 30s, 72°C 1min | 559 | 5′- ACG**G**TA**C**GCGCCG **G**AG**A**CAGAATG-3′ | [28] |
| | | | **Alternative primers:**<br>**CAP59LF** 5′ GTGAACAA GCTGCGGC 3′<br>**CAP59LR** 5′ GGATTCAG TGTGGTGGAAGA 3′ | 35 cycles: 94°C 30s, 60°C 30s, 72°C 1min | | | [current study] |
| *GPD1* | Glyceraldehyde-3-phosphate dehydrogenase | 7 | **GPD1F** 5′ CCACCGAACCC TTCTAGGATA 3′<br>**GPD1R** 5′ CTTCTTGGCA CCTCCCTTGAG 3′ | 94°C 3min; 35 cycles: 94°C 45s, 63°C 1min, 72°C 2min<br>**Alternative conditions:**<br>12 cycles; 62 − 56°C step-down 2°C every 2 cycles 95°C 3 min; 95°C 30 sec, 62 −56°C 30 s, 72°C 1 min; followed by 25 cycles: 95°C 30 s, 56°C 30 s, 72°C 1 min | 543 | 5′- GGTTTCGGTACGG GACCC**T**GCCAA-3′ | [28] |
| *LAC1* | Laccase | 8 | **LAC1F** 5′ AACATGTTCCCT GGGCCTGTG 3′<br>**LAC1R** 5′ ATGAGAATTG AATCGCCTTGT 3′ | 94°C 3min; 30 cycles: 94°C 30s, 58°C 30s, 72°C 1min<br>**Alternative conditions:**<br>30 cycles: 95°C 30s, 50°C 30s, 72°C 1min | 469 | 5′- GTAAGTA**T**CAGCT C**AA**GCTA**A**ACA**-**3′ | [28] |
| *PLB1* | Phospholipase | 12 | **PLB1F** 5′ CTTCAGGCGGA GAGAGGTTT 3′<br>**PLB1R** 5′ GATTTGGCGT TGGTTTCAGT 3′ | 94°C 3min; 30 cycles: 94°C 45s, 61°C 45s, 72°C 1min<br>**Alternative conditions:**<br>12 cycles; 62 −56°C step-down 2°C every 2 cycles 95°C 3 min; 95°C 30 s, 62 −56°C 30 s, 72°C 1 min; followed by 25 cycles: 95°C 30 s, 56°C 30 s, 72°C 1 min | 532 | 5′- TGT**T**AC**T**TGGATT **C**TGGAACATCG-3′ | [23] |
| *SOD1* | Cu, Zn superoxide dismutase | 5 | **Primers for *C.n.***<br>**SOD1CNF** 5′AAGCCTCT CATCCATATCTT 3′<br>**SOD1CNR** 5′TTCAACCAC GAATATGTA 3′<br>**Primers for *C.g.***<br>**SOD1CGF** 5′ GATCCTCAC GCCATTACG 3′<br>**SOD1CGR** 5′ GAATGATG CGCTTAGTTGGA 3′ | 94°C 3min; 35 cycles: 94°C 30s, 52°C 30s, 72°C 1.5min | 700 | 5′ - **C**CACGTGCTC**GCA** CC**TGTCAA**TGC-3′ | [46] |

| Gene locus | Gene product | Chromo-some location[a] | Primer name and sequence (If not specified differently primers listed will work for *C.n.* and *C.g.*) | Amplification conditions | No. of bases analysed (bp)[a] | Analysed sequence fragment, start (5′) and end (3′) points[a] | Ref. |
|---|---|---|---|---|---|---|---|
| | | | **Alternative primers for *C.n.*:**<br>**SOD1-f** 5′ TCTAATCGAAA TGGTCAAGG 3′<br>**SOD1-r** 5′ CGCAGCTGTT CGTCTGGATA 3′ | 12 cycles; 62 −56°C step-down 2°C every 2 cycles 95°C 3 min; 95°C 30 sec, 62 −56°C 30 sec, 72°C 1 min; followed by 25 cycles: 95°C 30 sec, 56°C 30 sec, 72°C 1 min | 535 | 5′ -ATCGCTCACCGCT GCCCATTGTCA-3′ | [23] |
| *URA5* | Orotidine monophosphate pyrophosphorylase | 8 | **URA5F** 5′ ATGTCCTCCCA AGCCCTCGAC 3′<br>**URA5R** 5′ TTAAGACCTCT GAACACCGTACTC 3′ | 94°C 3min; 35 cycles: 94°C 45s, 63°C 1min, 72°C 2min<br>**Alternative conditions:**<br>30 cycles: 94°C 45s, 63°C 1min, 72°C 2min (*C.n.*)<br>26 cycles: 94°C 30 s, 68°C 30s, 72°C 30s (*C.g.*)<br>**or:**<br>30 cycles: 95°C 3 min; 95°C 30 sec, 63°C 30 sec, 72°C 1min | 601 | 5′ - TTTTCGGCAACTCT TGGAA**A**GCTC-3′ | [16] |
| *IGS1* | Ribosomal RNA intergenic spacer | 2 | **IGSF** 5′ ATCCTTTGCAGA CGACTTGA 3′<br>**IGSR** 5′ GTGATCAGTGC ATTGCATGA 3′ | 94°C 3min; 35 cycles: 94°C 30s, 60°C 30s, 72°C 1min<br>**Alternative conditions:**<br>30 cycles: 94°C 30s, 56°C 30s, 72°C 1min | 723 | 5′ - TAAGCCCTTGTTA**A** AGAT**T**TA**TTG**-3′ | [23] |

Note:

[a]The sequences of the genome of strain H99 (*C. neoformans* var. *grubii*, VNI) at the Broad Institute (http://www.broad.mit.edu) were used as the master sequences. Nucleotide bases shown in bold typeface denote nucleotide bases that could vary between the different molecular types.