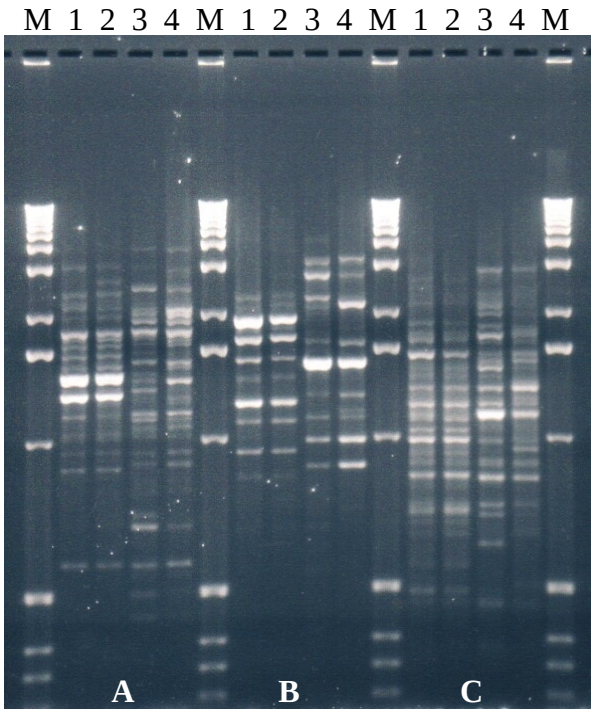
MLST typing as discussed at 3th TIMM, Torino, Italy, 29.10.2007

Present: Teun Boekhout, Massimo Cogliati, Maria Carmela Esposto, Matthew Fisher, June Kwon-Chung, Anastasia Letvintseva, Wieland Meyer

Nomenclature:
It was agreed between all present to use the VNI-VNIV and VGI - VGIV nomenclature (Wieland Meyer) since it correlates to the current species concept of two species and represents the global structure (based on more then 2000 global isolates) of the *Cryptococcus* species complex analysed, with *C. neoformans* var. *grubii* (serotype A = VNI) being the most prevalent molecular type.

Standard strains:
June Kwon-Chung suggested to use Wieland's standard strains as global standards, but also include H99 as an alternative standard for VNI and B3501 as an alternative standard for VNIV.

The argument from June was, that according to her M13 fingerprint analysis those alternative isolates for VNI and VNIV were identical. Based on this discussion we carried out the fingerprinting for those strains last week. According to our results the pair of WM148 and H99 (both VNI) and the other pair WM629 and B3501 (both are VNIV) are not identical using the following primers for PCR fingerprinting: M13, $(GACA)_4$ and $(GTG)_5$ (see below).



M = 1kb GIBCO-BRL marker
1 = WM 148 (VNI standard)
2 = H99
3 = WM 629 (VNIV standard)
4 = B 3501

A = PCR fingerprinting with the primer M13
B = PCR fingerprinting with the primer $(GACA)_4$
C = PCR fingerprinting with the primer $(GTG)_5$

Similarly sequencing using only 3 genes has shown that WM 148 and H99 have the same sequence types but WM 629 and B3501 have different sequence types for the three genes sequenced (see below).

Sequence types (based on results obtained in our lab (W Meyer) taking into account the numbering form Anastasia and James Fraser)

	Molecular Type	**URA5**	**PLB1**	**ACT1**
WM 148	**VNI**	**34**	**13**	**32**
H99	**VNI**	**38**	**18**	**33**
WM 629	**VNIV**	**24**	**16**	**24**
B3501	**VNIV**	**28**	**15**	**22**

In addition Teun suggested to include also existing type cultures for the other molecular types as far as they are available.

The above results show that those isolates are not identical but represent equally the molecular type VNI or VNIV. I agree with June and Teun, that we should as much as possible include already existing type cultures (Teun to supply the info and the strains for the other cultures) in view of a universal standardization and possible future description of further species, which would have to be based on the original type cultures.

-> Wieland should deposit the standard strains at the CBS and the ATCC.

- ⇨ We have already deposited the strains at the CBD, for CBS numbers please see below
- ⇨ We will look into the ways of how to submit our strains to ATCC
- ⇨ Teun and June should do the same for the other type culture strains

Standard strains agreed on during the meeting:

Cryptococcus neoformans

Cryptococcus neoformans* var. *grubii

VNI (Meyer) = AFLP1 (Boekhout) = VN6 (VN5) (Viviani)
WM 148 = CBS 10085 clinical isolate from Sydney, Australia, serotype A, CSF
Alternative isolate: H99

VNII (Meyer) = AFLP1A (Boekhout) = VN7 (Viviani)
WM 626 = CBS 10083 clinical isolate from Sydney, Australia, serotype A, CSF

AD hybrid

VNIII (Meyer) = AFLP3 (Boekhout) = VN3 + VN4 (Viviani)
WM 628 = CBS 10080 clinical isolate Melbourne, Australia, serotype AD, CSF

Cryptococcus neoformans* var. *neoformans

VNIV (Meyer) = AFLP2 (Boekhout) = VN1 (VN2) (Viviani)
WM 629 = CBS 10079 clinical isolate Melbourne, Australia, serotype D, blood
Alternative isolate: B3501

Cryptococcus gattii

VGI (Meyer) = AFLP4 (Boekhout)
WM 179 = CBS 10078 clinical isolate Sydney, Australia, serotype B, CSF

VGII (Meyer) = AFLP6 (Boekhout)
WM 178 = CBS 10082 clinical isolate Sydney, Australia, serotype B, Lung

VGIII (Meyer) = AFLP5 (Boekhout)
WM 175 = CBS 10081 tree hollow, San Diego, CA, USA, serotype B

VGIV (Meyer) = AFLP7 (Boekhout)
WM 779 = CBS 10101 veterinary isolate from Cheetah, South Africa, serotype C

Teun/Ferry could you please insert the alternative strains as you would suggest from the existing type cultures! And send the relevant strains out to all of us?

MLST typing scheme:

The following genes have been selected based on the results obtained in the studies by Anastasia, James Fraser, Wieland and Matthew, with a special emphasis on the number of different sequence types obtained and the possibility to use the same primers with all 8 major molecular types of the *Cryptococcus* species complex.

PCR condition are given as they have been used bye our lab (Wieland). We may need a consensus for this between different labs. This may differ between labs since we all use different water, buffer, Taq polymerase, etc.

MLST Locus 1: CAP59

Primers: **CAP59F 5' – TCC GCT GCA CAA GTG ATA CCC – 3'**
CAP59R 5' – CTC TAC GTC GAG CAA GTC AAG – 3'

Ref. Fraser JA, Giles SS, Wenink EC, Geunes-Boyer SG, Wright JR, Diezmann S, Allen A, Stajich JE, Dietrich FS, Perfect JR, Heitman J. Nature. 2005 Oct 27;437(7063):1360-4.

PCR Conditions:

In 50µL of PCR reaction

100 ng DNA
1X buffer
2 mM $MgCl_2$
50 ng reverse primer
50 ng forward primer
0,2 mM dNTP
2,5 U Taq polymerase

PCR cycles:

94ºC	3 min	
94ºC	30 sec	30X
54ºC	30 sec	
72ºC	30 sec	
72ºC	10 min	

MLST Locus 2: GPD1

Primers: **GPD1F 5' - CCA CCG AAC CCT TCT AGG ATA - 3'**
GPD1R 5' - CTT CTT GGC ACC TCC CTT GAG - 3'

Ref. Fraser JA, Giles SS, Wenink EC, Geunes-Boyer SG, Wright JR, Diezmann S, Allen A, Stajich JE, Dietrich FS, Perfect JR, Heitman J. Nature. 2005 Oct 27;437(7063):1360-4.

PCR Conditions:

In 50μL of PCR reaction

100 ng DNA
1X buffer
2 mM $MgCl_2$
50 ng reverse primer
50 ng forward primer
0,2 mM dNTP
2,5 U Taq polymerase

PCR cycles:

94ºC	3 min	
94ºC	45 sec	35X
63ºC	1 min	
72ºC	2 min	
72ºC	10 min	

MLST Locus 3: IGS1

Primers: IGSF 5' - ATC CTT TGC AGA CGA CTT GA - 3'
IGSR 5' - GTG ATC AGT GCA TTG CAT GA - 3'

Ref. Anastasia P. Litvintseva, Rameshwari Thakur, Rytas Vilgalys, Thomas G. Mitchell. Multilocus Sequence Typing Reveals Three Genetic Subpopulations of *Cryptococcus neoformans* var. *grubii* (Serotype A), Including a Unique Population in Botswana. Genetics. April 2006 (172): 2223–2238.

PCR Conditions:

In 50μL of PCR reaction

100 ng DNA
1X buffer
2 mM $MgCl_2$
50 ng reverse primer
50 ng forward primer
0,2 mM dntp
2,5 U Taq polimerase

PCR cycles:

94ºC	3 min	
94ºC	30 sec	35X
60ºC	30 sec	
72ºC	1 min	
72ºC	10 min	

MLST Locus 4: LAC1

Primers: **LAC1F 5'- AAC ATG TTC CCT GGG CCT GTG – 3'**
LAC1R 5' – ATG AGA ATT GAA TCG CCT TGT – 3'

Ref. Fraser JA, Giles SS, Wenink EC, Geunes-Boyer SG, Wright JR, Diezmann S, Allen A, Stajich JE, Dietrich FS, Perfect JR, Heitman J. Nature. 2005 Oct 27;437(7063):1360-4.

PCR Conditions:

In 50µL of PCR reaction

100 ng DNA
1X buffer
2 mM $MgCl_2$
50 ng reverse primer
50 ng forward primer
0,2 mM dNTP
2,5 U Taq polymerase

PCR cycles:

94ºC	3 min		
94ºC	30 sec	}	30X
58ºC	30 sec		
72ºC	1 min		
72ºC	10 min		

MLST Locus 5: PLB1

Primers: **PLB1CNF 5' – CTT CAG GCG GAG AGA GGT TT – 3'**
PLB1CNR 5' – GAT TTG GCG TTG GTT TCA GT – 3'

Ref. Anastasia P. Litvintseva, Rameshwari Thakur, Rytas Vilgalys, Thomas G. Mitchell. Multilocus Sequence Typing Reveals Three Genetic Subpopulations of *Cryptococcus neoformans* var. *grubii* (Serotype A), Including a Unique Population in Botswana. Genetics. April 2006 (172): 2223–2238.

PCR Conditions:

In 50µL of PCR reaction

100 ng DNA
1X buffer
2 mM $MgCl_2$
50 ng reverse primer
50 ng forward primer
0,2 mM dNTP
2,5 U Taq polymerase

PCR cycles:

94ºC	3 min		
94ºC	45 sec	}	30X
61ºC	45 sec		
72ºC	1 min		
72ºC	7 min		

MLST Locus 6: SOD1

Primers: **SOD1F 5' – TCT ATT CGA AAT GGT CAA GG – 3'**
SOD1R 5' – CGC AGC TGT TCG TCT GGA TA – 3'

Ref. Anastasia P. Litvintseva, Rameshwari Thakur, Rytas Vilgalys, Thomas G. Mitchell. Multilocus Sequence Typing Reveals Three Genetic Subpopulations of *Cryptococcus neoformans* var. *grubii* (Serotype A), Including a Unique Population in Botswana. Genetics. April 2006 (172): 2223–2238.

PCR Conditions:

In 50µL of PCR reaction

100 ng DNA
1X buffer
2 mM $MgCl_2$
50 ng reverse primer
50 ng forward primer
0,2 mM dNTP
2,5 U Taq polymerase

PCR cycles:

94ºC	3 min		
94ºC	45 sec	}	35X
60ºC	1 min		
72ºC	1 min		
72ºC	10 min		

MLST Locus 7: URA5

Primers: **URA5 5' - ATGTCCTCCCAAGCCCTCGAC – 3'**
SJ101 5' – TTAAGACCTCTGAACACCGTACTC – 3'

Ref. Wieland Meyer, Alexandra Castañeda, Stuart Jackson, Matthew Huynh, Elizabeth Castañeda, and the IberoAmerican Cryptococcal Study Group. Molecular Typing of IberoAmerican *Cryptococcus neoformans* Isolates. Emerging Infectious Diseases. 2003 February (9):189-195.

PCR Conditions:

In 50µL of PCR reaction

100 ng DNA

1X buffer
2 mM $MgCl_2$
50 ng reverse primer
50 ng forward primer
0,2 mM dNTP
2,5 U Taq polymerase

PCR cycles:

94ºC	3 min		
94ºC	45 sec	}	35X
63ºC	1 min		
72ºC	2 min		
72ºC	10 min		