Molecular Identification of Fungi

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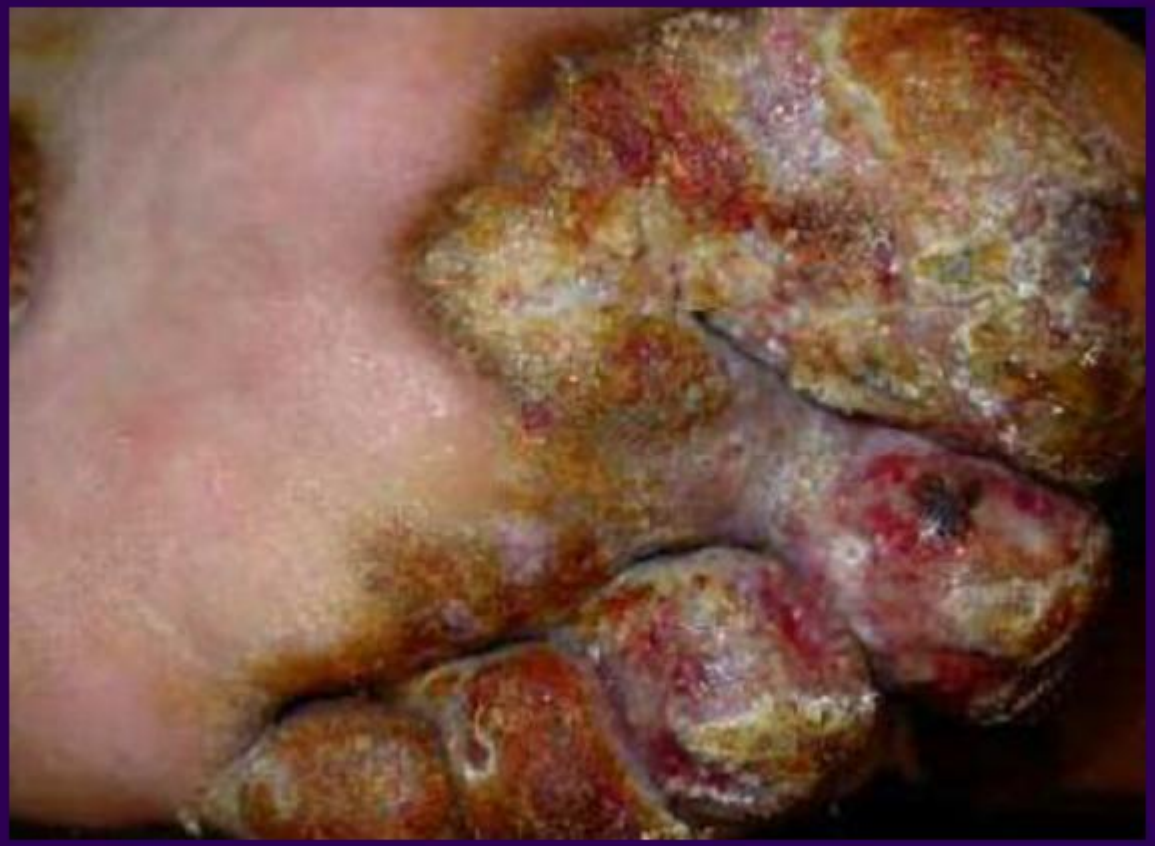
NEQAS USER DAY, November 2008

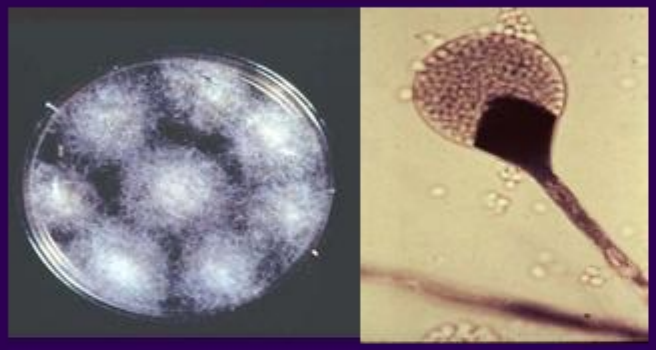


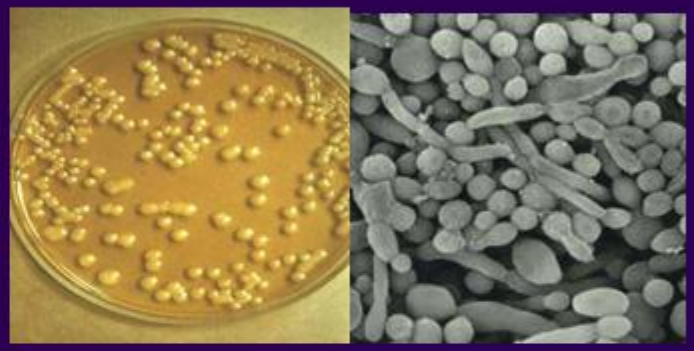
Athletes foot (*T. rubrum*) in a chronic granulomatous disease patient.

5 year history of microscopy-positive culture negative ?Dermatophyte infection largely unresponsive to antifungal therapy (Durant *et al*. *Med. Mycol*. 2008).

The Fifth Kingdom - Fungi

Includes moulds and yeasts :





In excess of 100 000 species of Fungus recognised; more than 700 species of yeast.

1500 new species described each year

> 600 species have been isolated from infections

(150-200 regularly isolated)

1 new/emerging pathogen per month

KINGDOM FUNGI - taxonomy

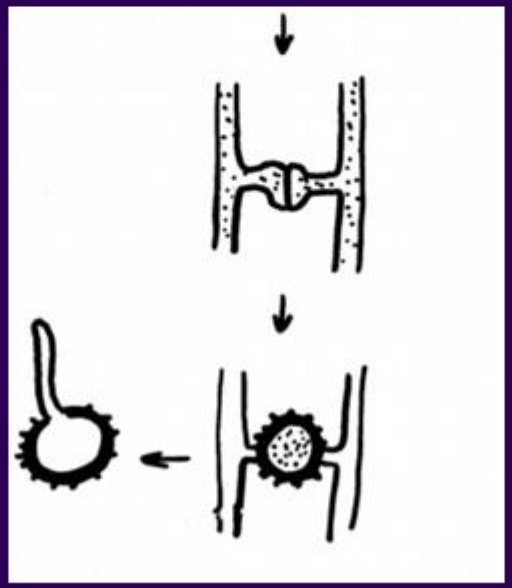
PHYLUM	ZYGOMYCOTA
PHYLUM	ASCOMYCOTA
PHYLUM	BASIDIOMYCOTA
(PHYLUM	DEUTEROMYCOTA)

Conventional identification and taxonomy (of moulds)

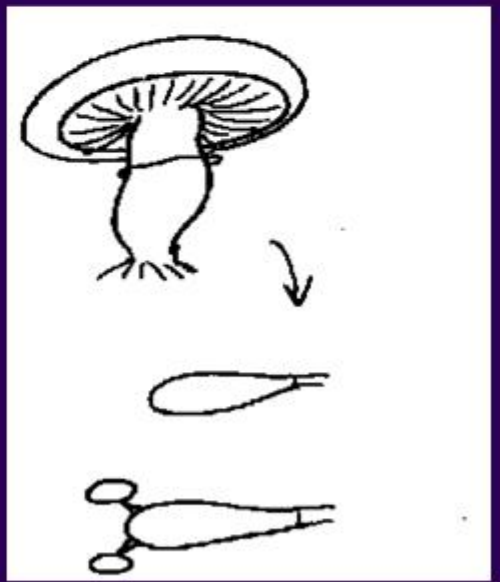
Historically relies on the characterisation of the structures produced by fungi during their sexual cycle :

Ascomycetes

Zygomycetes



Basidiomycetes



Female mould with nice fruiting bodies seeks male with huge conidia for raunchy time on Sabouraud's agar.....

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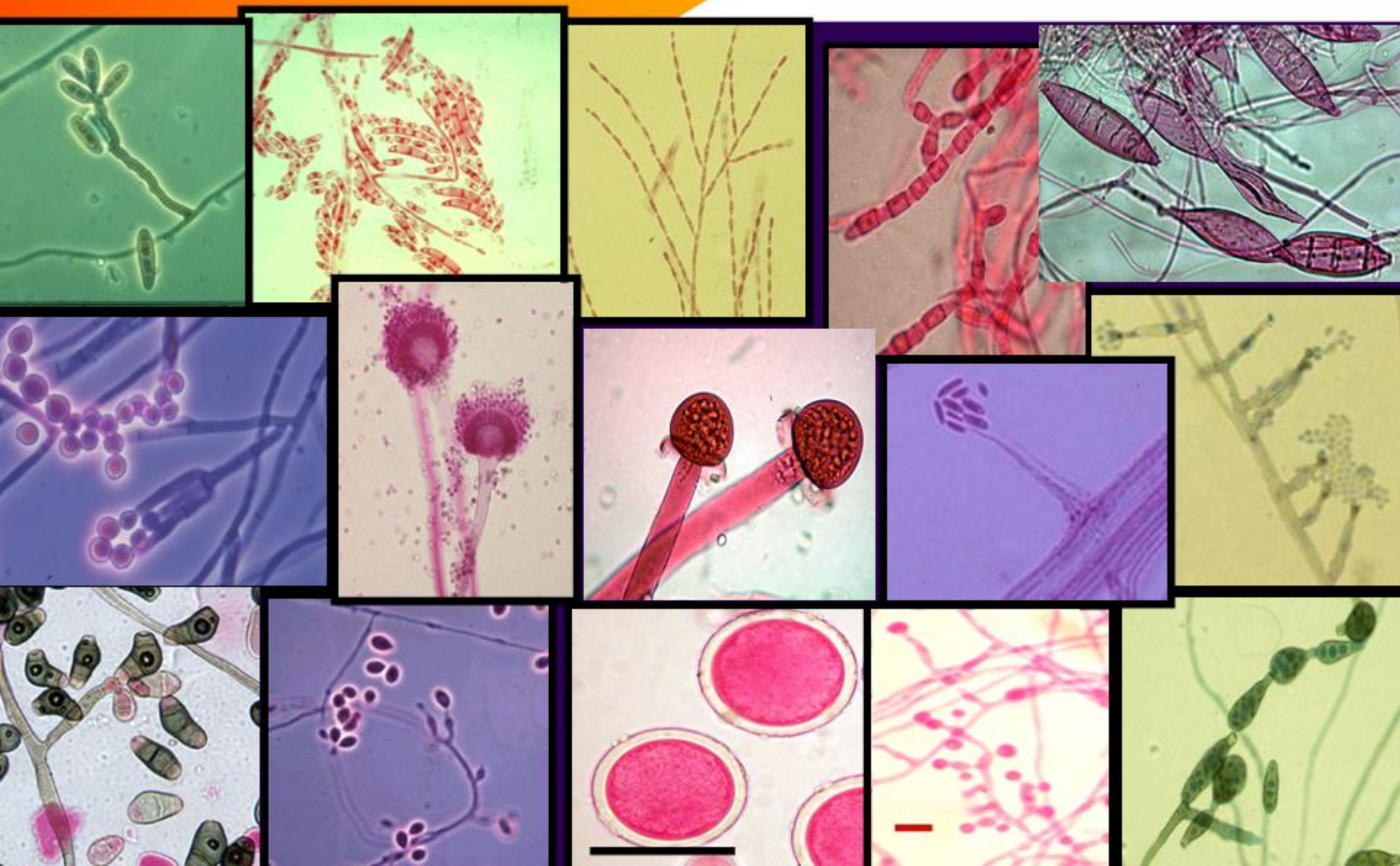
Unfortunately, many fungi can not be induced to enter a sexual cycle in the lab:

- Lack of a compatible mating partner (usually a clinical isolate is a single mating type)
- Incorrect growth conditions (natural substrates/host)

Instead, identification usually relies on the production of asexual spores (conidia).

Deuteromycetes or "imperfect fungi"

Some asexual spores (conidia)



Why do we need molecular identification methods?

- Can't grow the fungus even though we can see them
- Unable to identify even if growing :

  Non-sporing moulds –frequently those isolated from deep sites. Rare fungi -virtually any fungus capable of growth at 37°C is potential pathogen in patients with impaired immunity.
- Fungal Description in identification keys:
  - Conidiogenous cells mostly determinate or percurrent but not sympodial, polyblastic or where monoblastic intercalary.
  - Condiophores very slender with a swollen vesicle at the apex, conidia clathrate or muriform, sometimes corniculate.
  - Stroma erumpent, infundibuliform
- Cryptic species, only distinguish using DNA
- Taxonomic affiliations for imperfect fungi **(no known sexual cycle)**

BUT HOW??

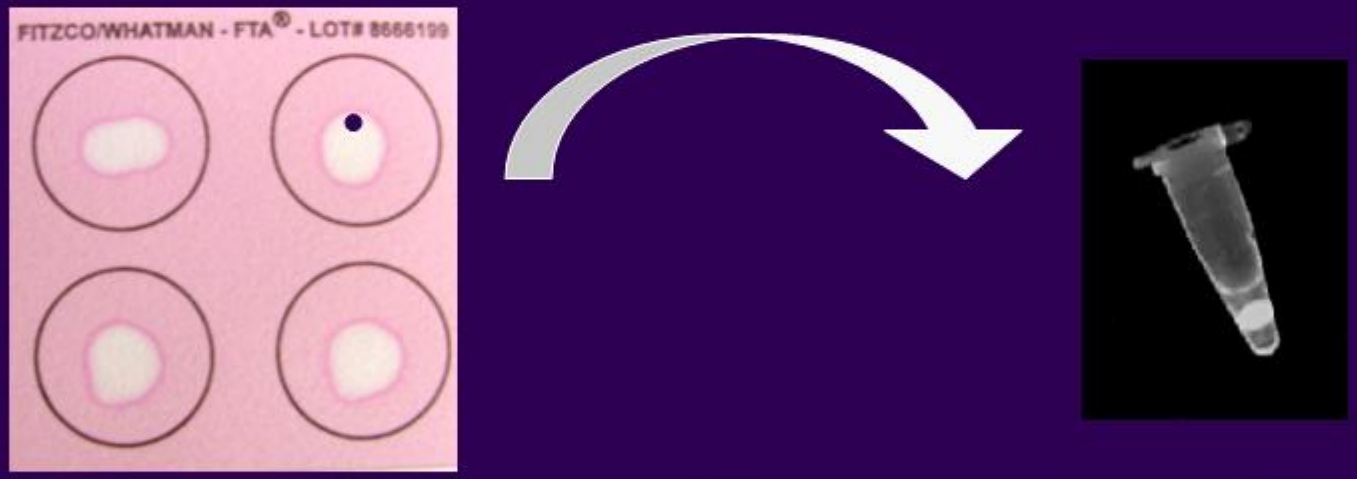
- Until recently, very laborious to extract fungal genomic DNA
- Inherent problems with breaking fungal cell walls and spores
- Significant quantities of potential PCR inhibitors
- Onerous techniques- many hours, expensive, multiple steps involving centrifugation/organic solvents etc.

FTA TECHNOLOGY FOR FUNGAL DNA EXTRACTION

- Cards are chemically treated filter paper composed of filtration matrix
- Denaturants and chelators- induce cell lysis- inactivate organism
- Nucleic acids become physically trapped in the fibres of the matrix
- Cards/nucleic acids can be stored at room temperature for long periods (>15 years to date for huDNA)

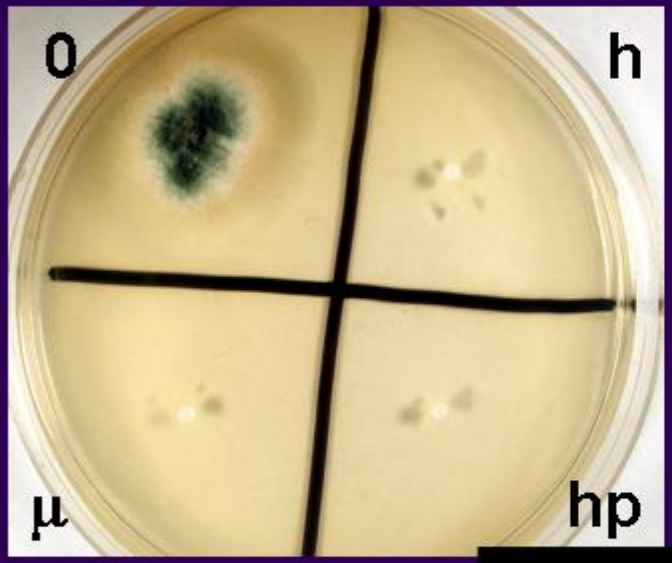
PROTOCOL

- **Apply homogenous aqueous suspension of yeast cells or mould spores and hyphal fragments directly to FTA matrix cards and dry cards**



- Filter punches removed from the area of the dried spot using a Micro Punch.
- The discs are transferred into a PCR tube, washed twice with Whatman FTA Purification reagent then twice with TE buffer and dried
- The washed and dried discs are then directly used for molecular detection.

PROTOCOL part 2

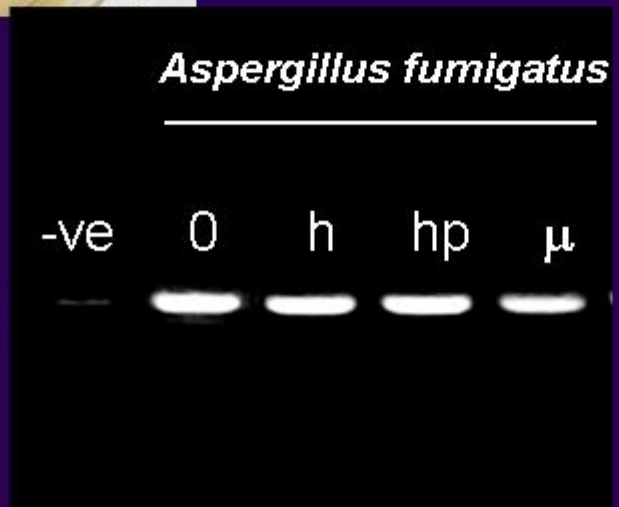


0 - suspension applied directly to FTA

h - suspension pre-treated 95°C 20min

hp- pre-treated 95°C 20min, proteinase K 15 min

μ– suspension applied, filter micro-waved 2x30 s



Success rates with FTA

2004-2006 :

Successful PCR from filters for 218/226 organisms tested

Included 38 species of yeast and 75 species of mould

Organisms tested covered ascomycetes, zygomycetes and basidiomycetes

Microwave treatment inactivated all organisms tested.

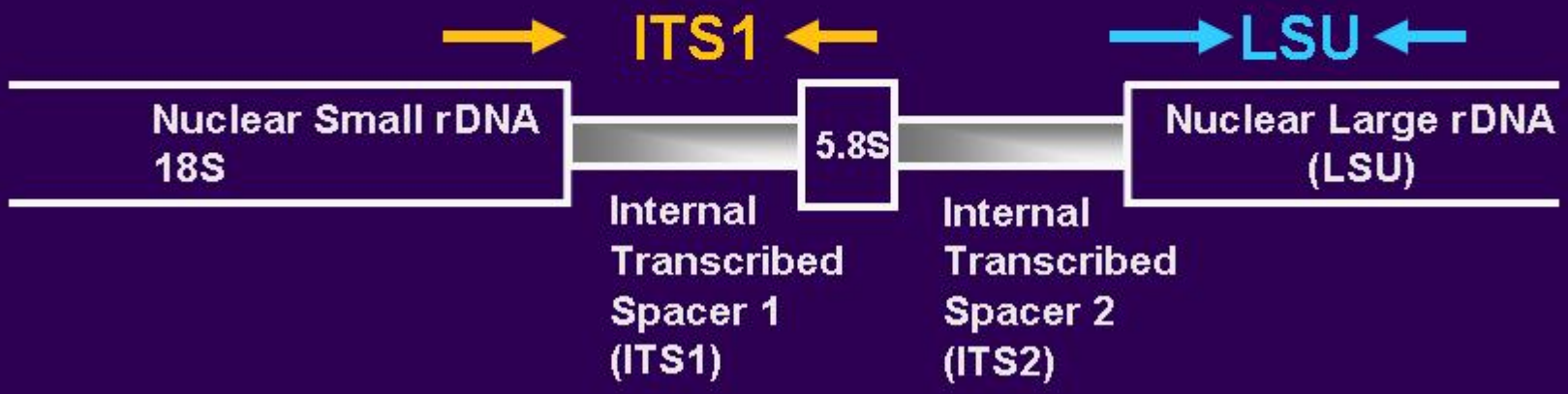
(Borman *et al.*, 2006 Med. Mycol. 44, 389-398)

Now tested in excess of 2000 isolates of mould and yeast
(70 species of yeast; 150 species of mould) with equivalent success rates.

Amplification Targets used for Fungi

- Should be highly conserved across species – "pan-fungal" PCR primers
- But be sufficiently variable to allow accurate identification to species level after sequencing of the PCR products

Nuclear Ribosomal Repeat – Multi-copy genes



Moulds to species level (for some genera)

Yeasts to species level
Moulds to genus level

Aspergillus fumigatus

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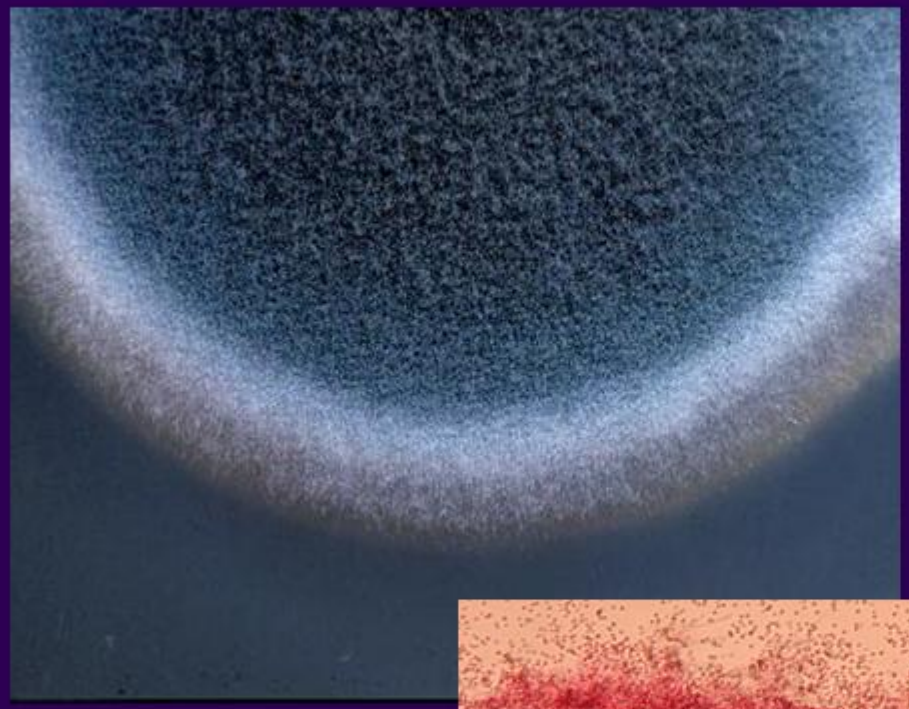

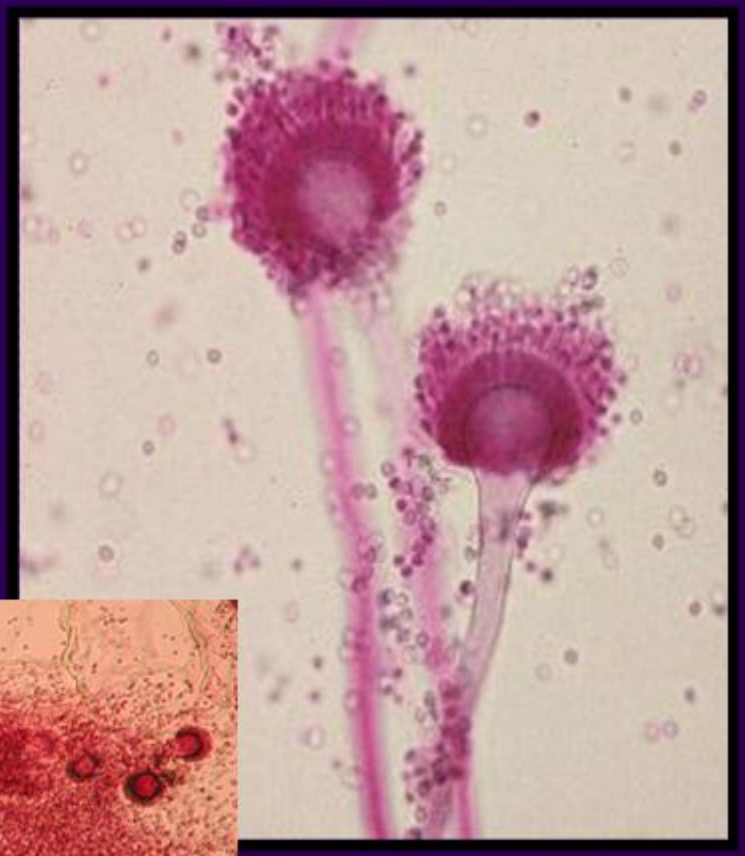

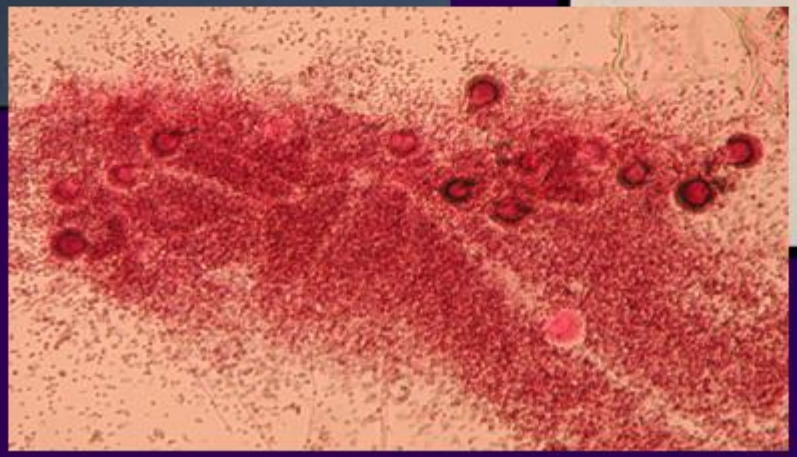

Clinically……

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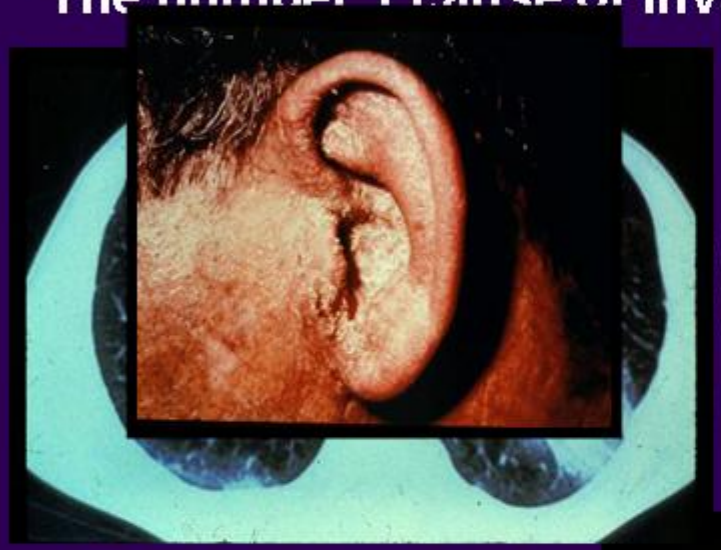
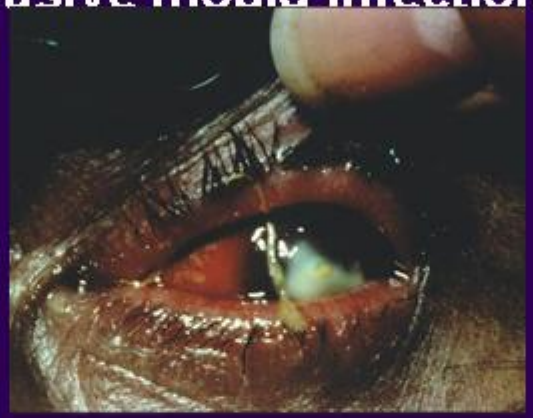
Otitis externa

Keratitis

The number 1 cause of invasive mould infections in neutropenic patients

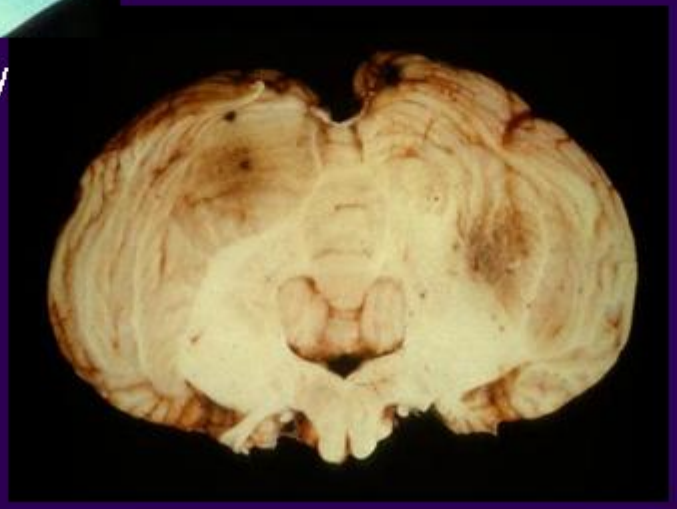


CT scan, invasive pulmonary Aspergillosis



Aspergillus sinusitis in a leukemic patient

Anaissie EJ. Aspergillus Audioconference, 1998.



Necrotic brain lesions

We used to think this was an easy fungus to ID !!

MIC_{90}	AMB	ITR	VOR	CAS
A. fumigatus	1	0.25	0.25	0.5
A. lentulus	1-2	1	1-2	2-16
A.thermomutans	1-2	1	2-4	0.03
A.udagawae	2-4	0.5	1	0.125

S.A. Balajee (Eukaryotic Cell, 2005; Eukaryotic Cell 2006)

Several cryptic species within *A. fumigatus* which can only be reliably identified by β-tubulin gene sequencing :

A. lentulus
A. thermomutans
A. udagawae

A. novofumigatus
A.fumigatiaffinis

Found in an analysis of "*A.fumigatus*" isolates that sporulate poorly and/or slowly

MRL – 50 isolates selected as poorly-sporing and/or aberrant–sequencing ITS1 and β-Tubulin

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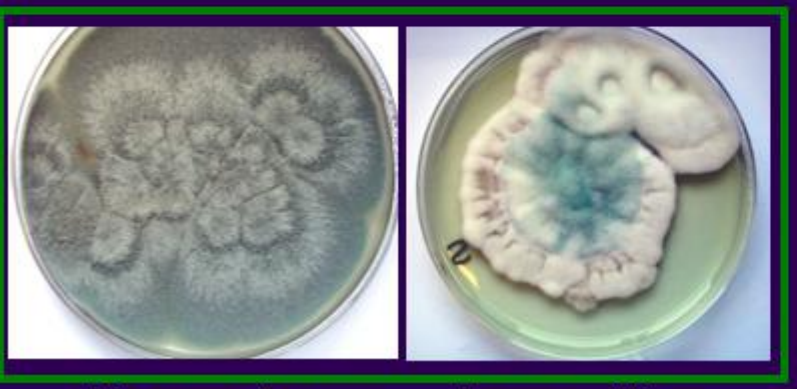
mut. helvola

var alba

All 100% identical to *A. fumigatus*



Neosartorya quadracinta

Aspergillus fumigatiaffinis

Aspergillus thermomutans

Neosartorya hiratsukae

Aspergillus fumigatus complex

A. brevipes
A. duricaulis
A. fumigatus
A. fumigatiaffinis
A. fumisynnematous
A. lentulus
A. novofumigatus
A. thermomutatus
(N. pseudofischeri)
A. turcosus
A. viridinutans
A. unilateralis
N. assulata
N. aureola
N. denticulate
N. fennelliae
N. fischeri
N. galapagensis
N. hiratsukae
N. quadricinta
N. udagawae

Molecular ID of unusual moulds:

the agents of dark grain mycetoma

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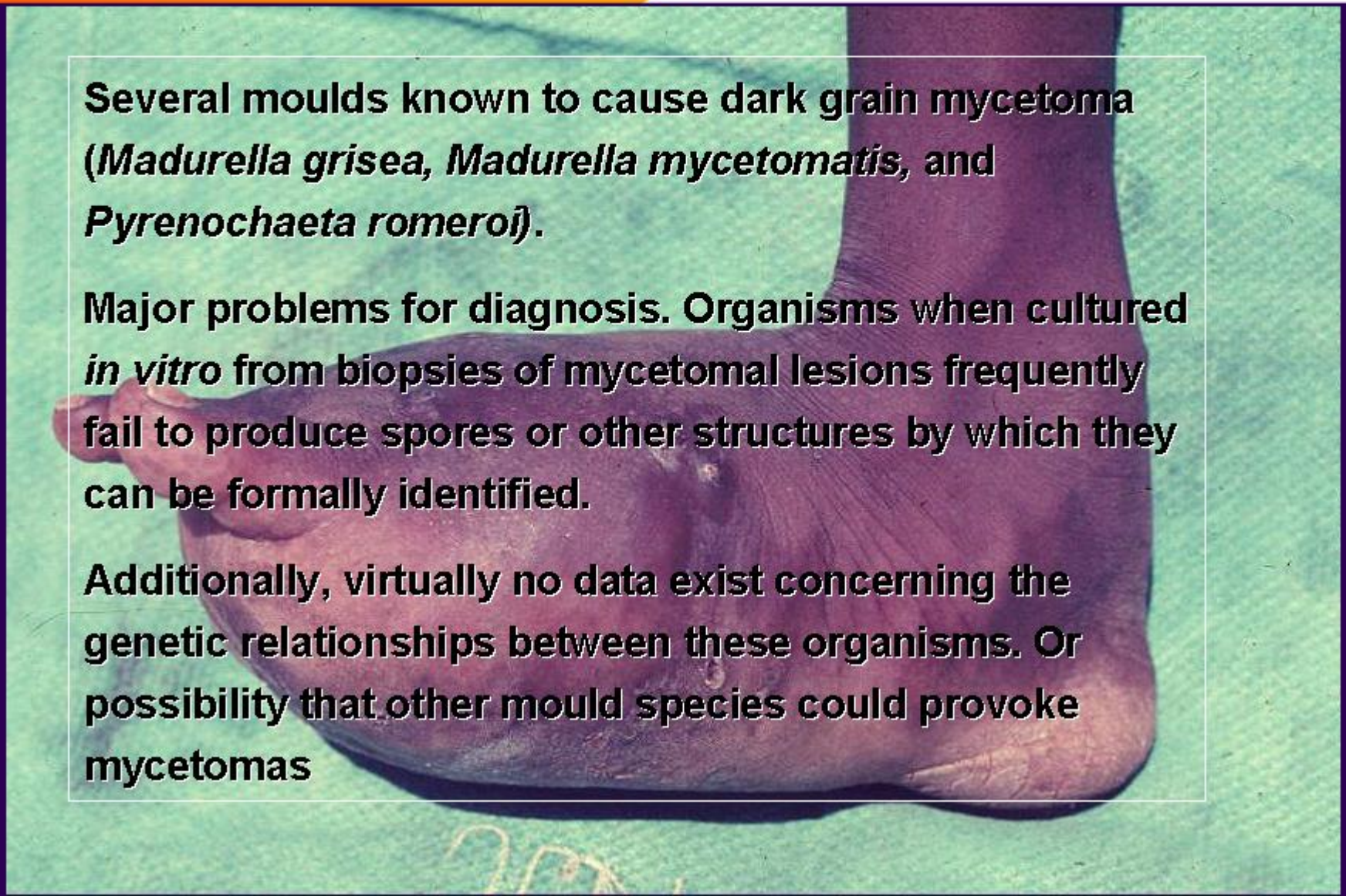
Fungal mycetoma:

Most infections occur in tropical areas but imported cases of fungal mycetoma do occur in the UK.

Infections follow the traumatic implantation of fungal spores or hyphal fragments present in soil or on plant material.

Destructive infection of the skin and subcutaneous tissues, which if untreated, progresses to involve muscle and bone.

Chemotherapy with antifungal drugs is often not effective - surgical methods, often involving amputation of the affected limb.

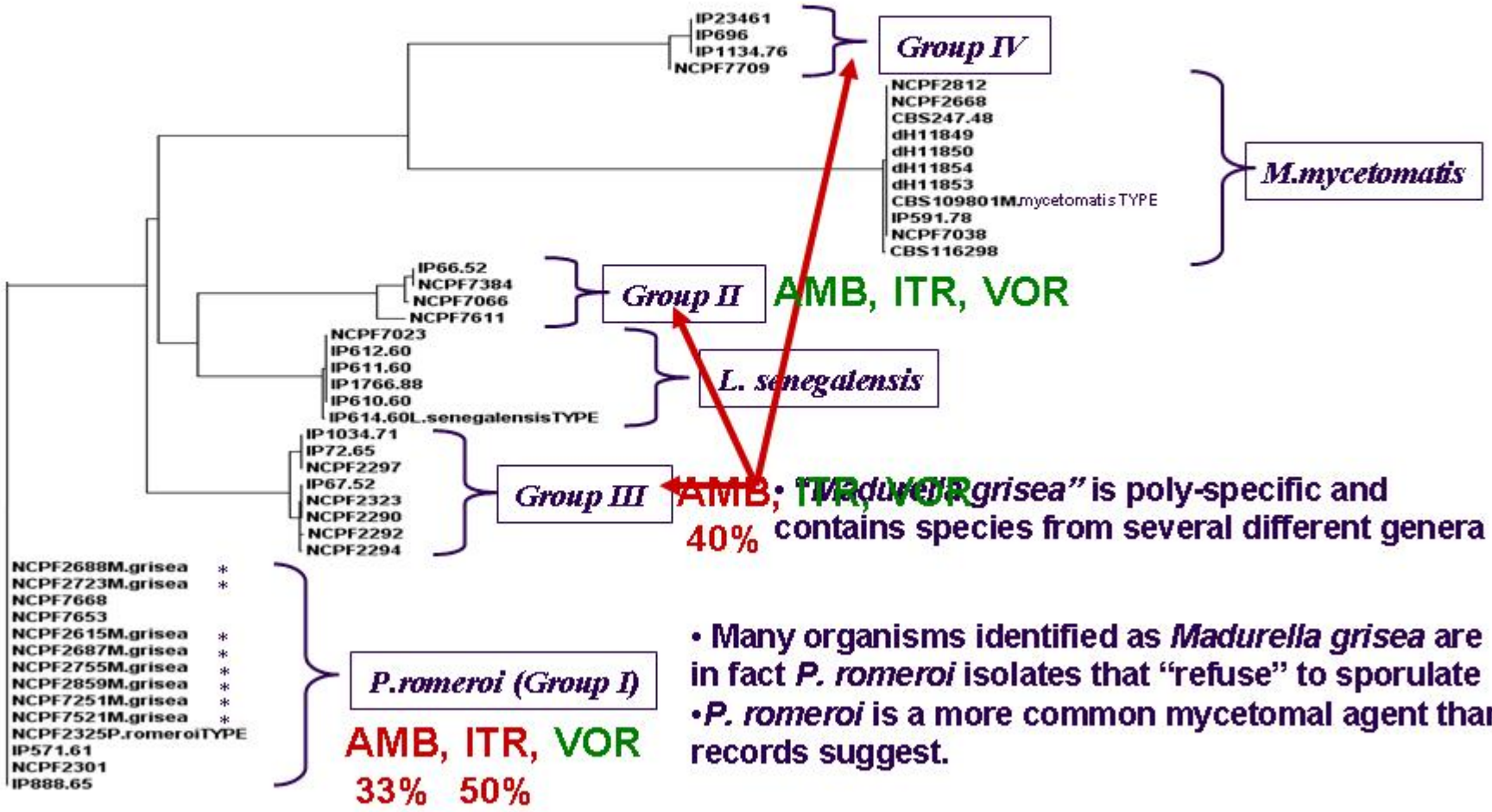


Several moulds known to cause dark grain mycetoma (*Madurella grisea, Madurella mycetomatis,* and *Pyrenochaeta romeroi)*.

Major problems for diagnosis. Organisms when cultured *in vitro* from biopsies of mycetomal lesions frequently fail to produce spores or other structures by which they can be formally identified.

Additionally, virtually no data exist concerning the genetic relationships between these organisms. Or possibility that other mould species could provoke mycetomas

Mycetoma continued……



• *"Madurella grisea"* is poly-specific and contains species from several different genera

• Many organisms identified as *Madurella grisea* are in fact *P. romeroi* isolates that "refuse" to sporulate

•*P. romeroi* is a more common mycetomal agent than records suggest.

(Miles *et al.,* in preparation)

Role of molecular techniques for clinical yeast isolate ID

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> 700 species of yeast described

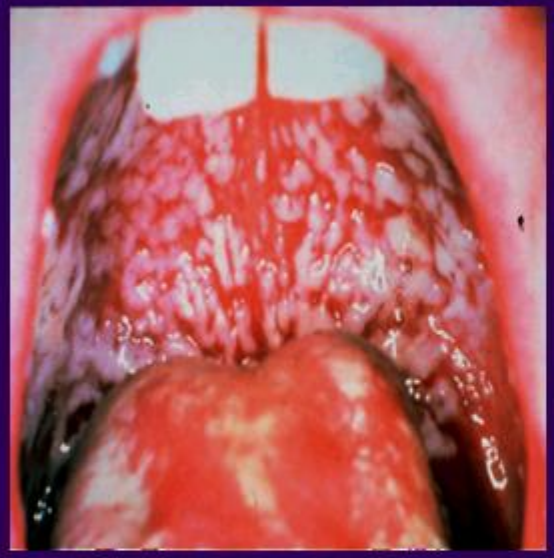
> 200 species (25 genera) isolated from human infections

Conventional (fermentation/assimilation) ID kits cover 30 - 40 most commonly encountered species

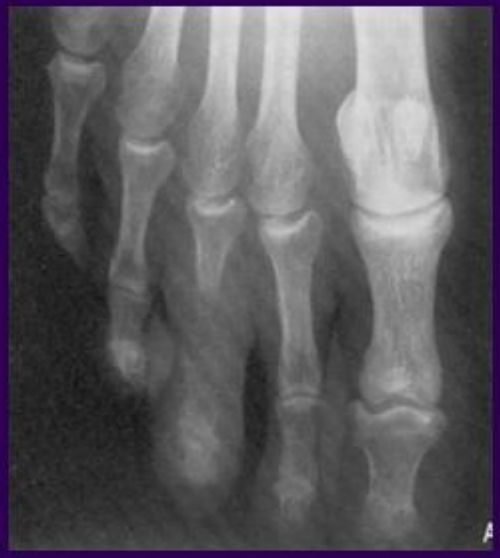
Kits don't work if the organism is misbehaving

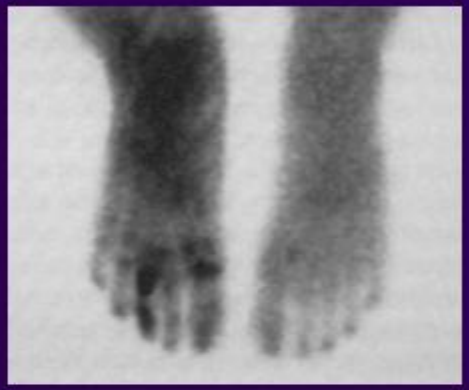
Many species-specific trends in antifungal susceptibility profiles

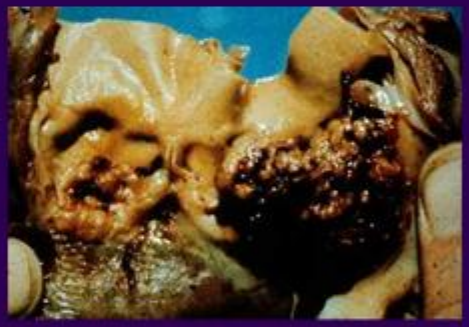
Candidosis

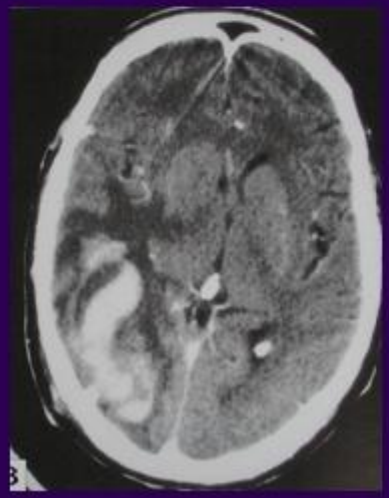


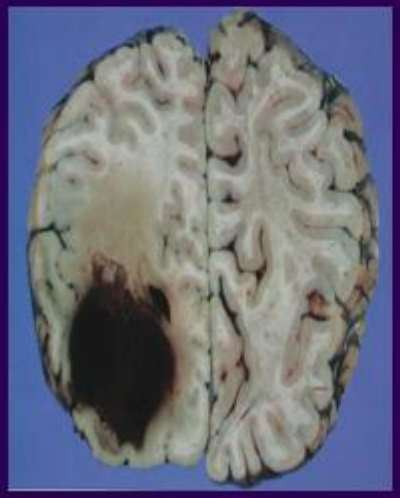
Mucosal infection







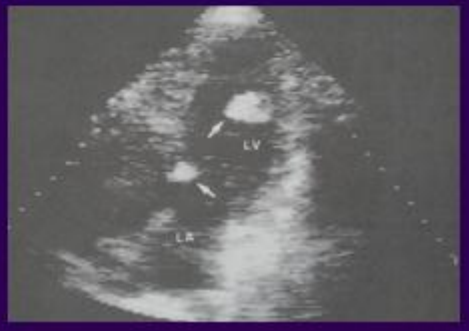




CT scan showing micro- and macro-abscesses, and haemorrhaging

PM findings of same patient (39yr old, ALL)

Estimated that 50% patients with disseminated Candidosis have CNS involvement.



Yeast ID at the MRL

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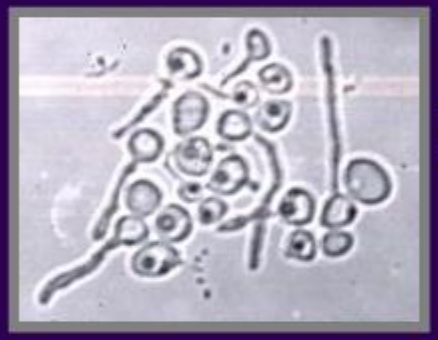
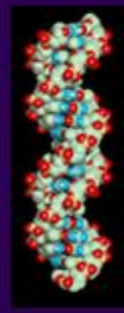


+ ve

C.albicans

- ve

API20C



FTA filters, PCR amplification D1D2, sequencing, BLAST analyses, phylogenetic comparisons MRL database

Yeast species identified 2004-2006 as a function of frequency of isolation

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Organism	Number received	Number requiring molecular ID	% requiring molecular ID
C. albicans	1192	7	0.6
C. glabrata	659	6	0.9
C.parapsilosis	430	3	0.7
C.tropicalis	156	0	0
C.lusitaniae	99	19	19.2
C.krusei	74	4	5.4
Cr.neoformans	64	3	4.7
S.cerevisiae	60	4	6.7
C.guilliermondii	53	19	35.8
M.pachydermatis	36	2	5.5
C.dubliniensis	33	3	9.1
Trichosporon sp.	23	4	17.4
Rhodotorula sp.	19	0	0
C.inconspicua	17	5	29.4
C.kefyr	15	4	26.7
C.famata	12	3	25
C.nivariensis	11	11	100
Geotrichum sp.	10	3	30
C.pelliculosa	8	2	25
C.fabianii	5	5	100
C.lipolytica	5	3	60
C.blankii	4	4	100
C.utilis	4	1	25
Cr. albidus	4	0	0
C.rugosa	3	1	33.3
C.norvegensis	3	3	100
C.pararugosa	3	3	100
C.catenulata	3	3	100
Kloeckera sp.	3	1	33.3
S.elongasporus	2	2	100
C.zeylanoides	2	2	100
C.eremophila	2	2	100
C.lambica	2	2	100
C.cilerii	1	1	100
C.boidinii	1	1	100
C.palmioleophila	1	1	100
C.freyschussii	1	1	100
C.magnoliae	1	1	100
C.viswanathii	1	1	100
C.haemulonii	1	1	100
C.pseudointermedia	1	1	100
C.pseudoglaebosa	1	1	100

>9/10 OK
>2/3 OK
<2/3 OK

Linton *et al.*, J. Clin Microbiol. 2007. 45; 1152-8

A common question (or criticism)

Q: Why bother identifying yeast isolates to species level ?

A: Epidemiology, surveillance and also antifungal choice (many species-specific trends in antifungal susceptibility profiles)

Q: Yes but you can perform antifungal susceptibility testing faster than you can arrive at a molecular identification

A: fair point / still needed for epidemiological studies / oh go away / **NOT ANY MORE WE HOPE**

Pyrosequencing® as a potential ultra-rapid yeast ID system

Perform a PCR reaction to amplify microbial DNA (RT-PCR for viral RNA). One of the PCR primers is biotinylated in order to create a single-stranded DNA template in the next step *(30 minutes - 2 hours)*.

Create single-stranded DNA from the PCR product using the Vacuum Prep Workstation and Tool *(15 minutes)*.

Run the Pyrosequencing reaction on the single-stranded DNA template. Nucleotides are dispensed in a cyclic manner, and each incorporation event generates a flash of light proportional to the number of nucleotides incorporated, displayed as a peak in the Pyrogram™. The sequence is base-called automatically *(1 minute/nucleotide; up to 96 samples in parallel)*.

Images courtesy of

Biotage

www.biotage.com

How does Pyrosequencing® work ??

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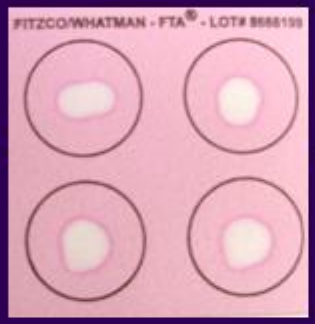
The Pyrosequencing reaction cascade system

The Pyrosequencing reaction cascade system

Pyrosequencing® of clinical yeast isolates

9 am



9.15

PCR ITS2
PyroMark Fungi
ASR kit

11.15

Pyrosequencing
96 samples=1h10

2 pm

2005-11-24

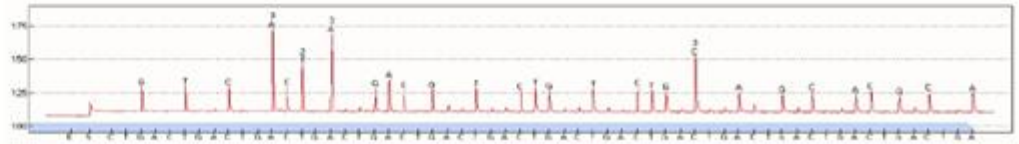
IdentiFire
Detailed Report

Biotage

Sample ID:	**3.11**
Well:	G4
PSQ run:	Pyro3
Entry ID:	CTGA
Sequence library:	Fungi Library 050929 100 v2 (2005-10-12, 2:50:04 PM)
Query sequence:	GTCAAACTTAAAGACGTCTG

Result: Candida glabrata gi|23953802|gb|AY139784.1| Candida glabrata strain NYSDOH 181-89 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; large subunit ribosomal RNA gene, partial sequence", Candida glabrata gi|5566466|gb|AF167993.1|AF167993 Candida glabrata internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence" **Score:** 100

Quality: Good
Information: Top result contains multiple hits (2).



Terms and Condition

Does Pyrosequencing® work ??
Organisms tested so far which give unique pyrosequencing profiles:

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ORGANISM	Pyrosequencing profile	Similarity	Number Tested
C. albicans	GTCAAAGTTT GAAGATATAC	20	22
C. dubliniensis	GTCAAAGTTT GAAGAataAa	16	14
C. lipolytica	GTCAAAcTTa aAAGAacaAC	14	2
C. tropicalis	GTCAAAGTTa tgAaATAaAt	14	33
Zygosaccharomyces bailii	GTCAAAcTTT GAgagTATtg	14	1
C. kefyr	GTCAAAcTTT GAgagTtTtg	13	15
C. pelliculosa	GTCAAAcTTT tAgttTATtg	13	10
C. famata	GTCAAAcTTg tttGtTATAt	13	3
C. parapsilosis	GTCgAAtTTg GAAGAagTtt	13	71
C. orthopsilosis	GTCgAAtTTg GAAGAatTtt	13	8
Saccharomyces cerevisiae	GTCAAAcTTT aAgaAcATtg	13	15
C. blankii	GTCAAAtTTT GgAGcggTtg	13	1
C. metapsilosis	GTCgAAtTTg GAAGAatgtt	12	4
Lloderomyces elongisporus	GTCgAAGTTT GAAatataga	12	2
*Kazachstania complex**	GTCAAAcTTa aAAGAacact	12	3
C. zeylanoides	GTCAAAcTTT GtttgTtgtt	11	1
C. nivariensis	GTCAAAcTTa aAgGtTcctg	11	26
C. glabrata	GTCAAAcTTa aAGacgtctg	10	47
C. norvegensis	GTCgAgcTTa GAtttaAaAa	10	4
C. guilliermondii	GTCAAAcTTg tttGgTtgtt	10	27
*C. fabianii/obtusa**	GTCAAAcTTa tgAagaAatt	10	9
C. utilis	GTCAAgcTTa GAAaggtgtt	10	1
C. lusitaniae	GgCgAAaTgT cgtGcTgTAa	10	59
Sporopachydermia cereana	GTCAAAGaTT tAgatctTtg	10	2
*Cryptococcus saitoi/friedmanii**	GcCAgAtgTT atgaATATta	10	1
C. inconspicua	GTCgAgcTTg attaAagTtC	9	11
*C. eremophila/lambica**	GTCgAgcTTa GttaAaAgtt	9	1
Filobasidiella uniguttulatum	GTCAgAtgTc aAAGtataca	9	1
C. krusei	GTCgAgcTTT ttgttgtctC	8	25
C. rugosa	aatAAcGTca aAgGgTccgt	7	4
Cryptococcus neoformans	GTCAAAcaaa aAgagatggt	7	16
Pichia membranifaciens	GTCgAgCTca atgatatatt	6	3

N=442

C. parapsilosis complex

(contains 3 distinct species [2 new ones] on basis of MLST using fragments from 11 genes; Tavanti *et al.*, 2005. *J.Clin.Microbiol.* 43; 284-292)

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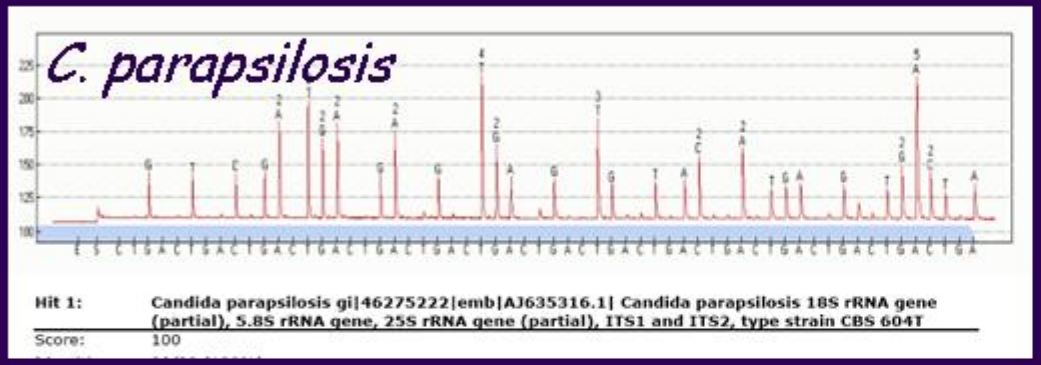
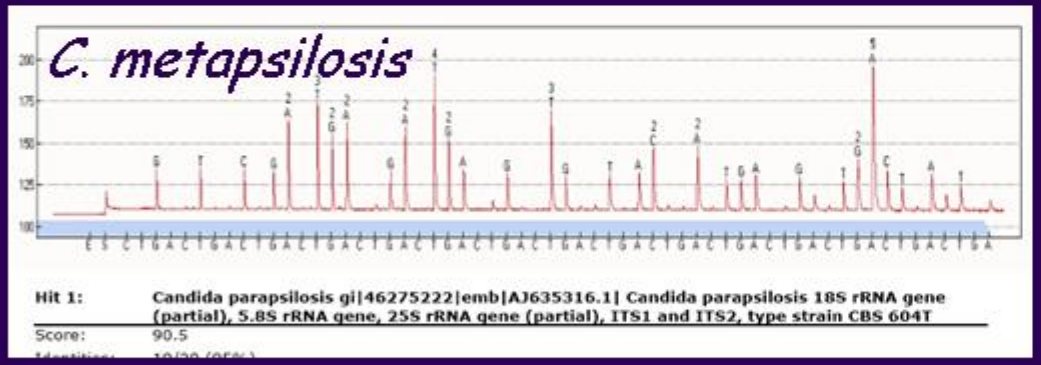


Score:	100
Identities:	20/20 (100%)
Gaps:	0/20 (0%)
E-value:	1.10e-013

```
Query    1  GTCGAATTTGGAAGAAGTTT 20
            ||||||||||||||||||||
Library  1  GTCGAATTTGGAAGAAGTTT 20
```



Score:	90
Identities:	19/20 (95%)
Gaps:	1/20 (5%)
E-value:	3.19e-010

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            |||||||||||||||||| |||
Library  1  GTCGAATTTGGAAGAA~GTT 19
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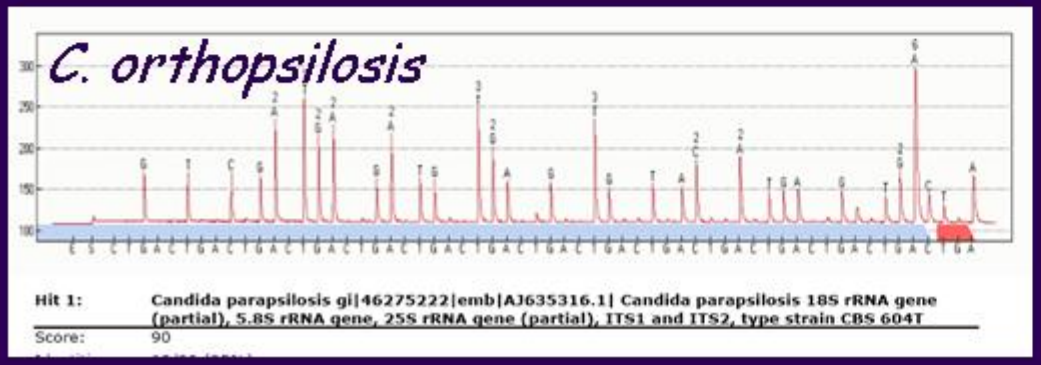


Score:	90.5
Identities:	19/20 (95%)
Gaps:	0/20 (0%)
E-value:	1.10e-013

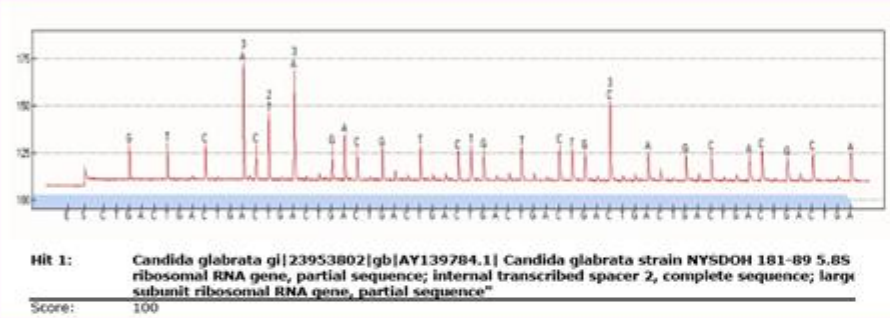
```
Query    1  GTCGAATTTGGAAGAATTTT 20
            |||||||||||||||||| |||
Library  1  GTCGAATTTGGAAGAAGTTT 20
```

C. nivariensis – an emerging yeast pathogen??

(Borman et *al.*, 2008. *J. Clin. Microbiol.* 46; 933-938.)

Health Protection Agency

C. glabrata

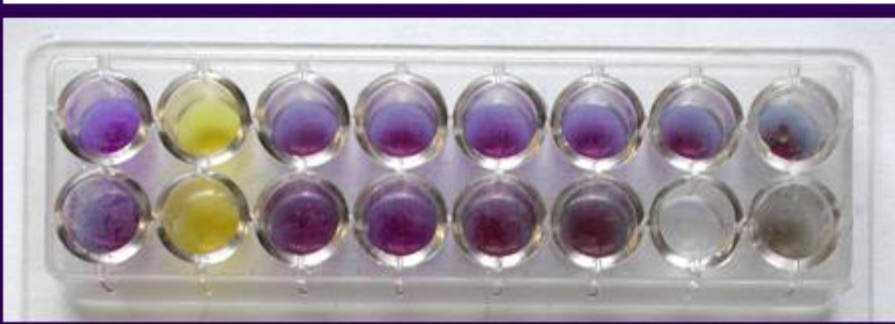


Hit 1: Candida glabrata gi|23953802|gb|AY139784.1| Candida glabrata strain NYSDOH 181-89 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; large subunit ribosomal RNA gene, partial sequence"

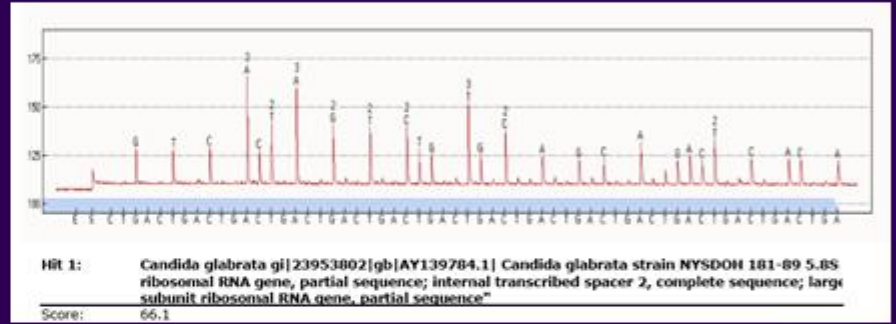
Score: 100

Score: 100
Identities: 20/20 (100%)
Gaps: 0/20 (0%)
E-value: 1.10e-013

```
Query    1  GTCAAACTTAAAGACGTCTG 20
            ||||||||||||||||||||
Library  1  GTCAAACTTAAAGACGTCTG 20
```



C. nivariensis 16 isolates in 2 years (17th most common *Candida* at MRL)



Hit 1: Candida glabrata gi|23953802|gb|AY139784.1| Candida glabrata strain NYSDOH 181-89 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; large subunit ribosomal RNA gene, partial sequence"

Score: 66.1

Score: 66.1
Identities: 18/22 (82%)
Gaps: 4/22 (18%)
E-value: 1.27e-004

```
Query    1  GTCAAACTTAAAG~~GTTCCTG 20
            |||||||||||||  ||  |||
Library  1  GTCAAACTTAAAGACGT~~CTG 20
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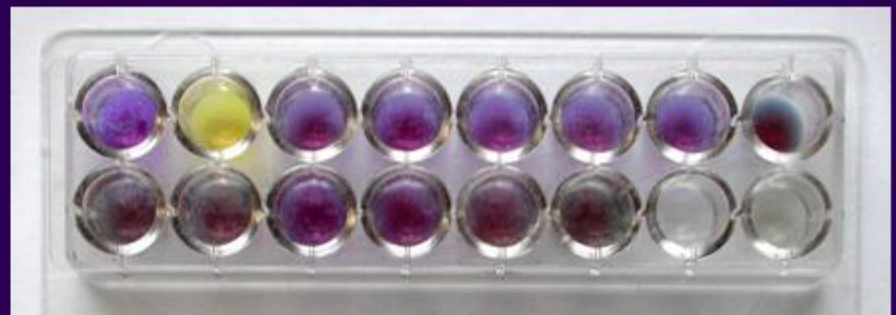

Potential clinical impact of *C.nivariensis*

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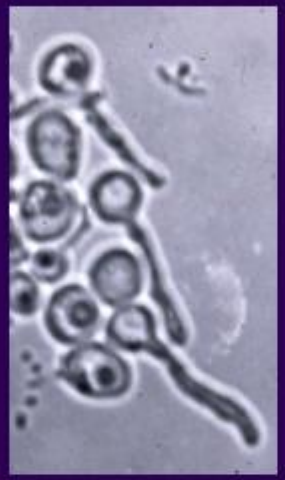
More than half of isolates received were cultured from deep, usually sterile body sites – indicative of systemic infection
(blood, biopsies, ascitic fluid, abscess)

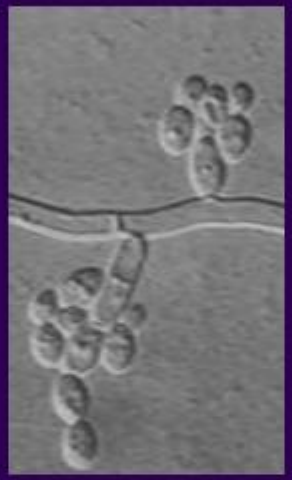
		AMB	ITR	VOR	FLU	FLY	POS	CAS
C. nivariensis	MIC_{90}	0.5	>16	4	>64	0.5	2	1
	MFC_{90}	2	>16	>16	>64	32	>16	2
C. glabrata	MIC_{90}	1	1	0.5	32	<0.1	4	2
	MFC_{90}	2	>16	>16	>64	<0.1	>16	>64

Pyrosequencing future plans

- In theory can identify yeast species by pyrosequencing in a ½ day
- Relatively inexpensive after equipment costs (60K) ~ £1-2 per sample
- The 40 or so yeast species tested to date yield unique profiles (3 species complexes)
- Develop Pyrosequencing assays for antifungal resistance mutations – ideally suited to any phenotype conferred by single well-defined point mutations
  (viral genotyping and resistance mutation studies)

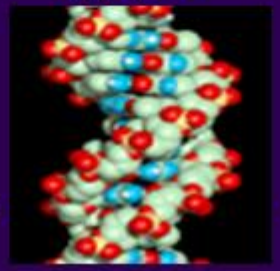
The Future





ITS2 PCR
PyroMark Fungi
ASR kit

ITS 2



Final Identification

Acknowledgments

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(Rebecca Petch)

NCTC, HPA, Colindale.

Haroun Shah

British Antarctic Survey, Cambridge.

Paul Bridge

Whatman International

Biotage

Institut Pasteur, Paris

Sybren de Hoog, CBS, Netherlands

Promoting professional mystique……..

Metschnikowia pulcherrima

Pseudochaetosphaeronema larense

Staphylotrichum coccosporum

Mycoleptodiscus indicus

Coniothyrium fuckelii

Dichotomophthoropsis nymphaearum

Colletotrichum gloeosporioides

Sporobolomyces salmonicolor

Trichophyton vanbreuseghemii