PCR-based Diagnosis of Dermatophytic Disease

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ISHAM Working Group

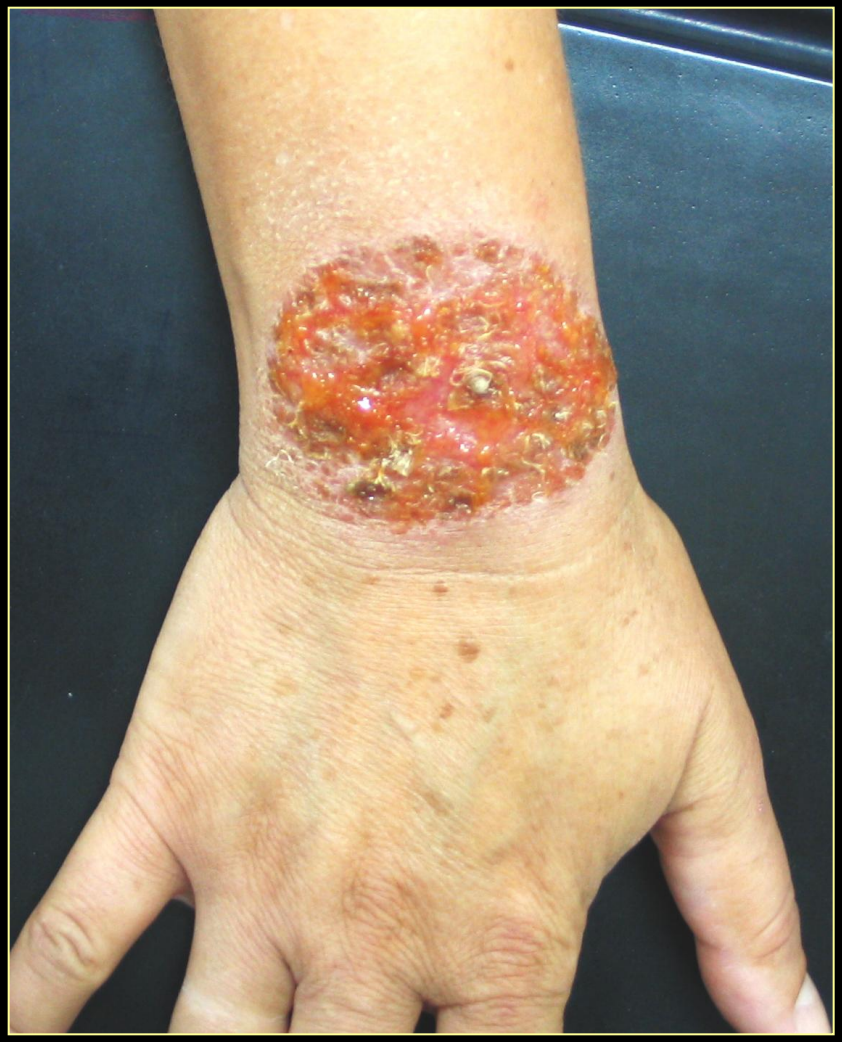
PCR-based diagnosis of Dermatophytic infections:

on the way to a consensus

Coordinators: Susan M. Abdel-Rahman, Michael Arabatzis, Jong-Soo Choi, Yvonne Graeser

Dermatophytes

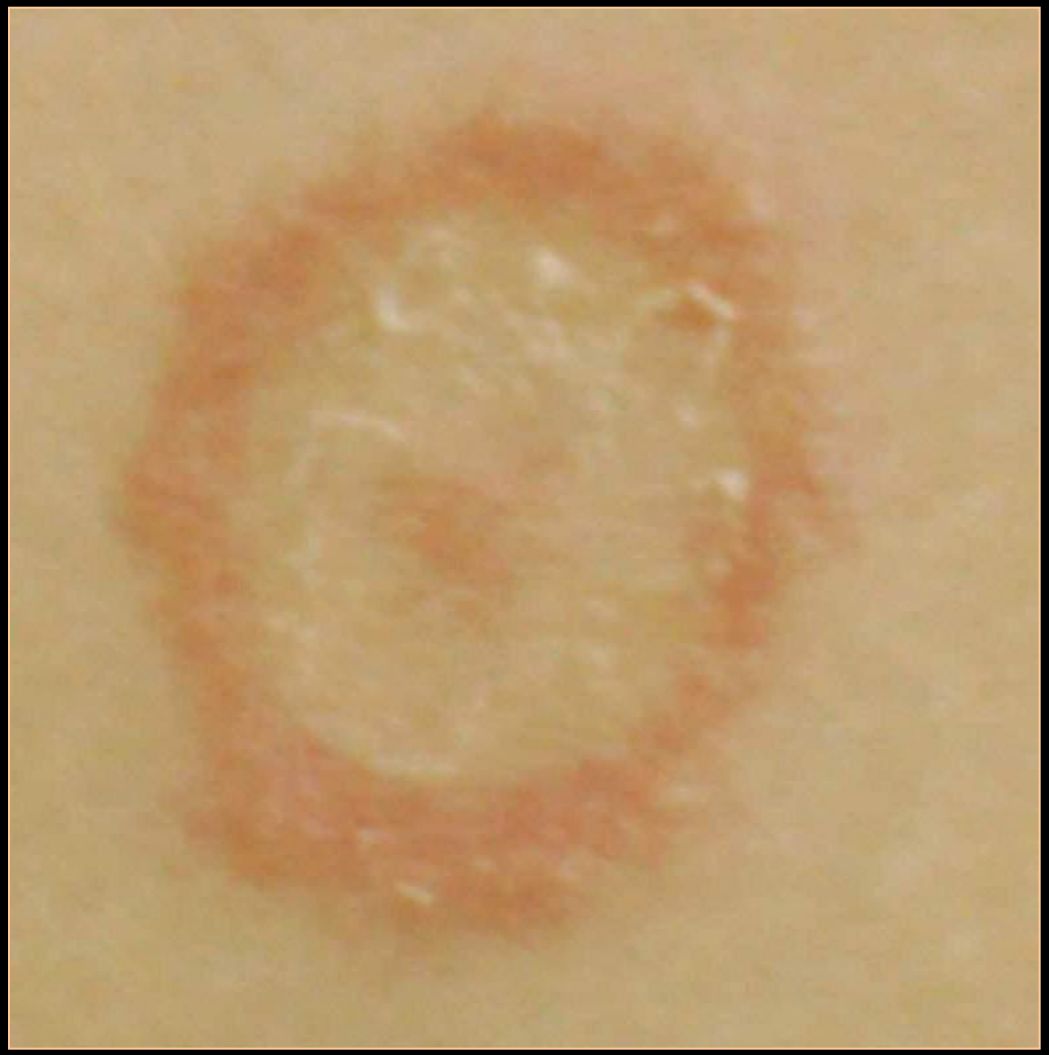
Primary Pathogens of Humans and Animals



Dermatophytes

Molds

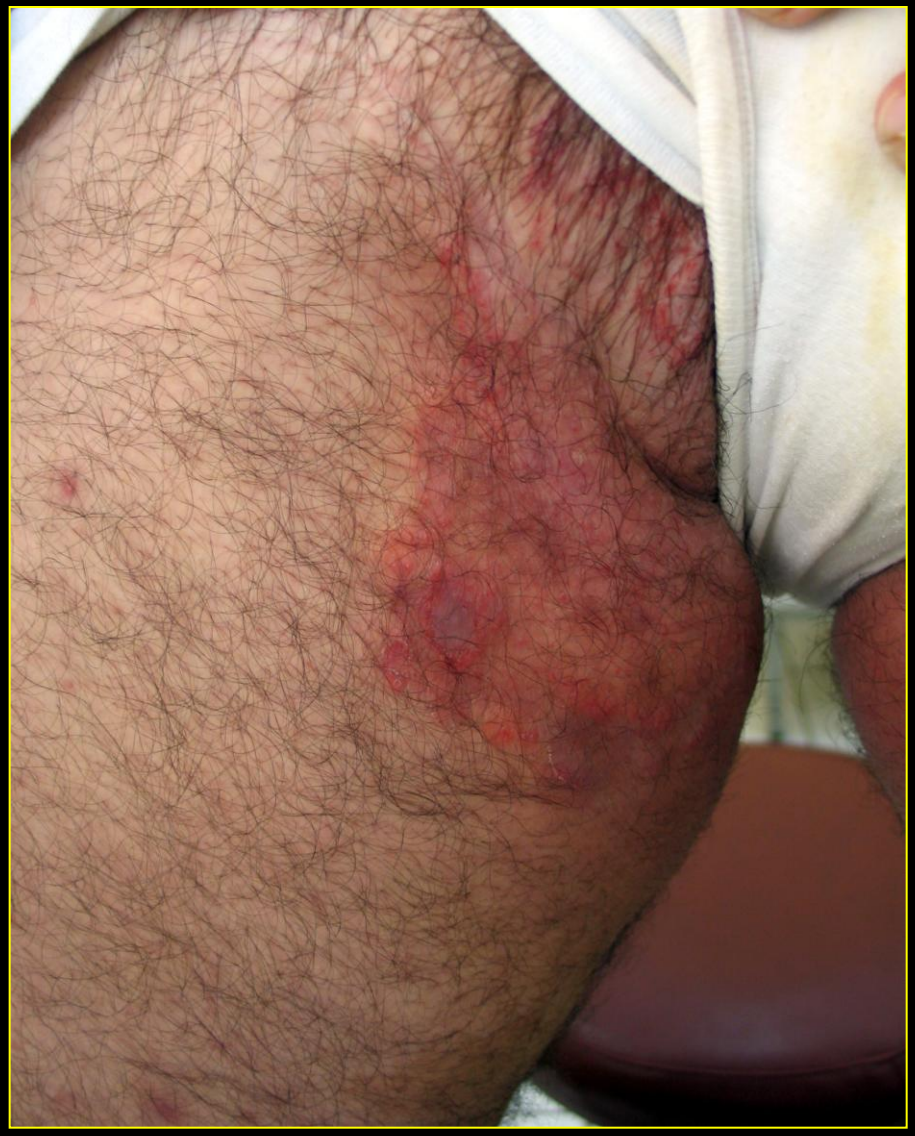
Keratinophilic Fungi

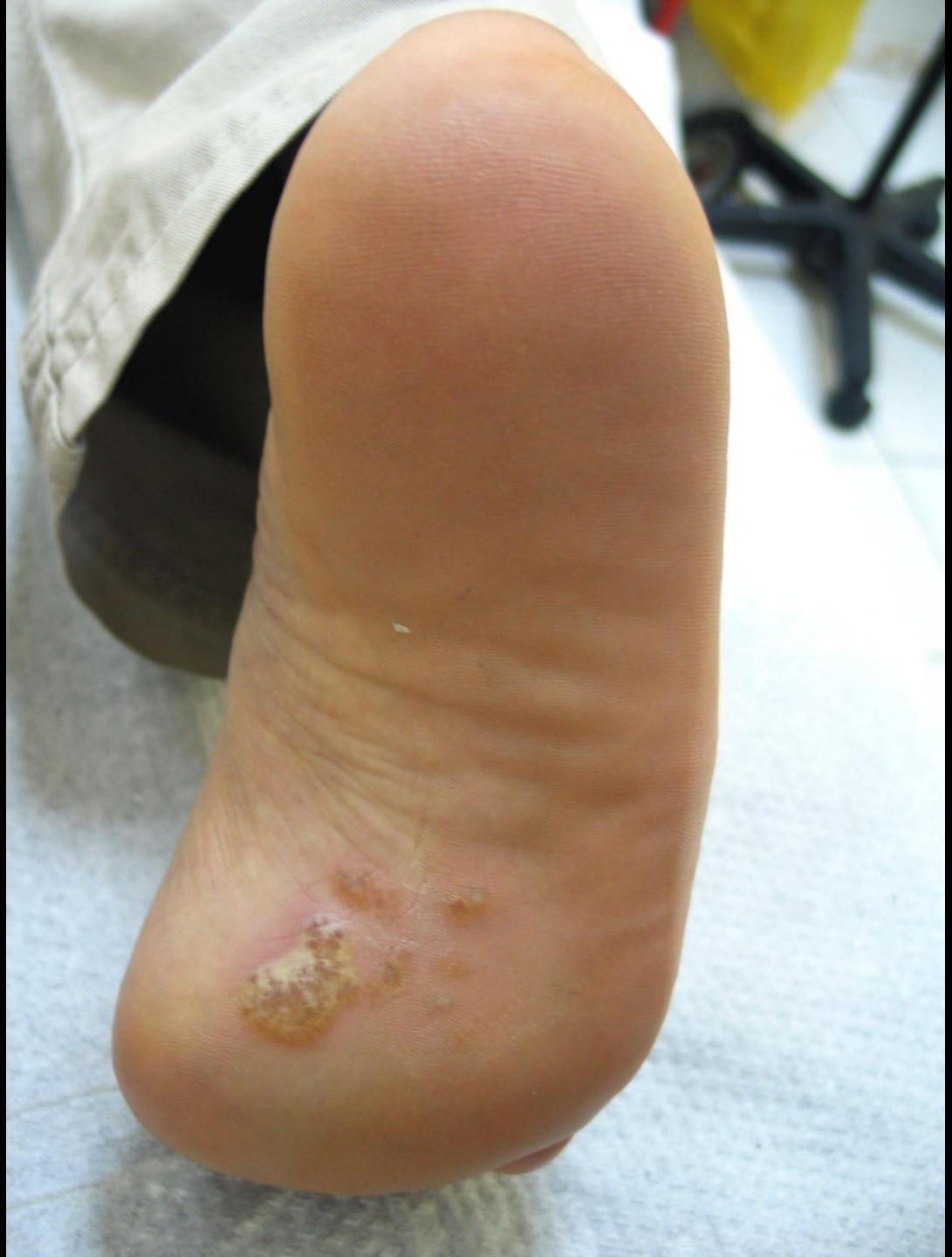


Human Lesion:
Tinea*

*Lat.: clothes moth

Tinea cruris





Tinea pedis

Subcutaneous forms of dermatophytoses

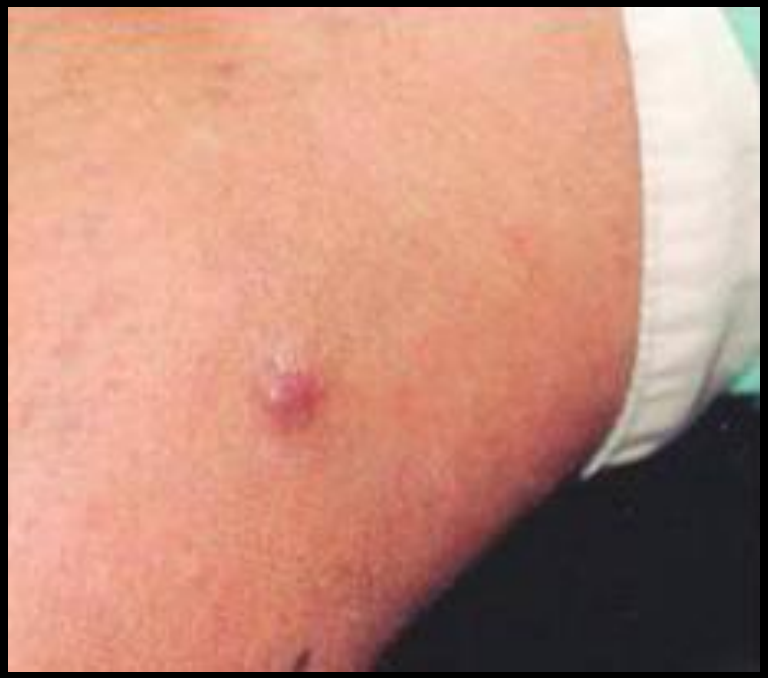
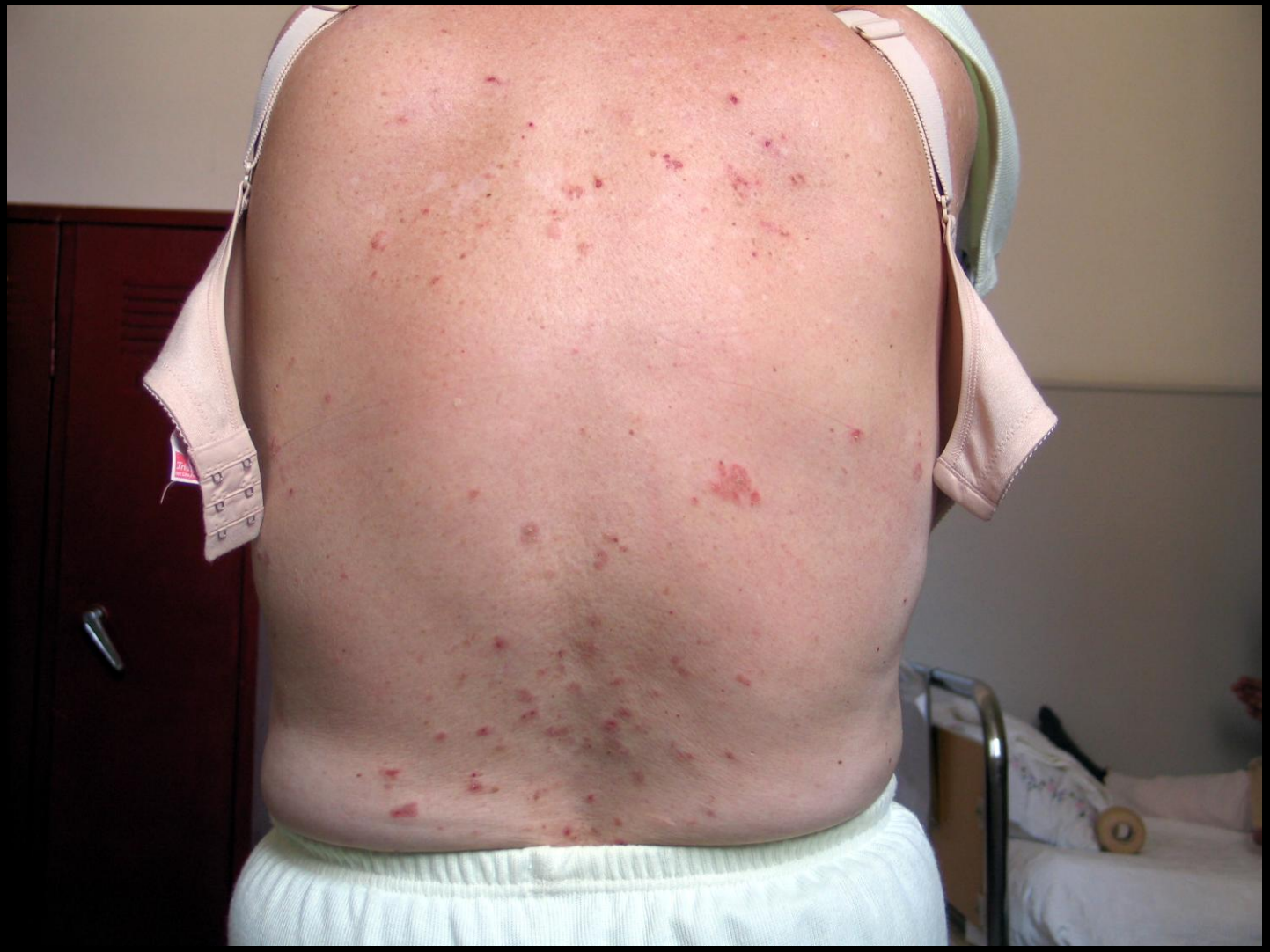
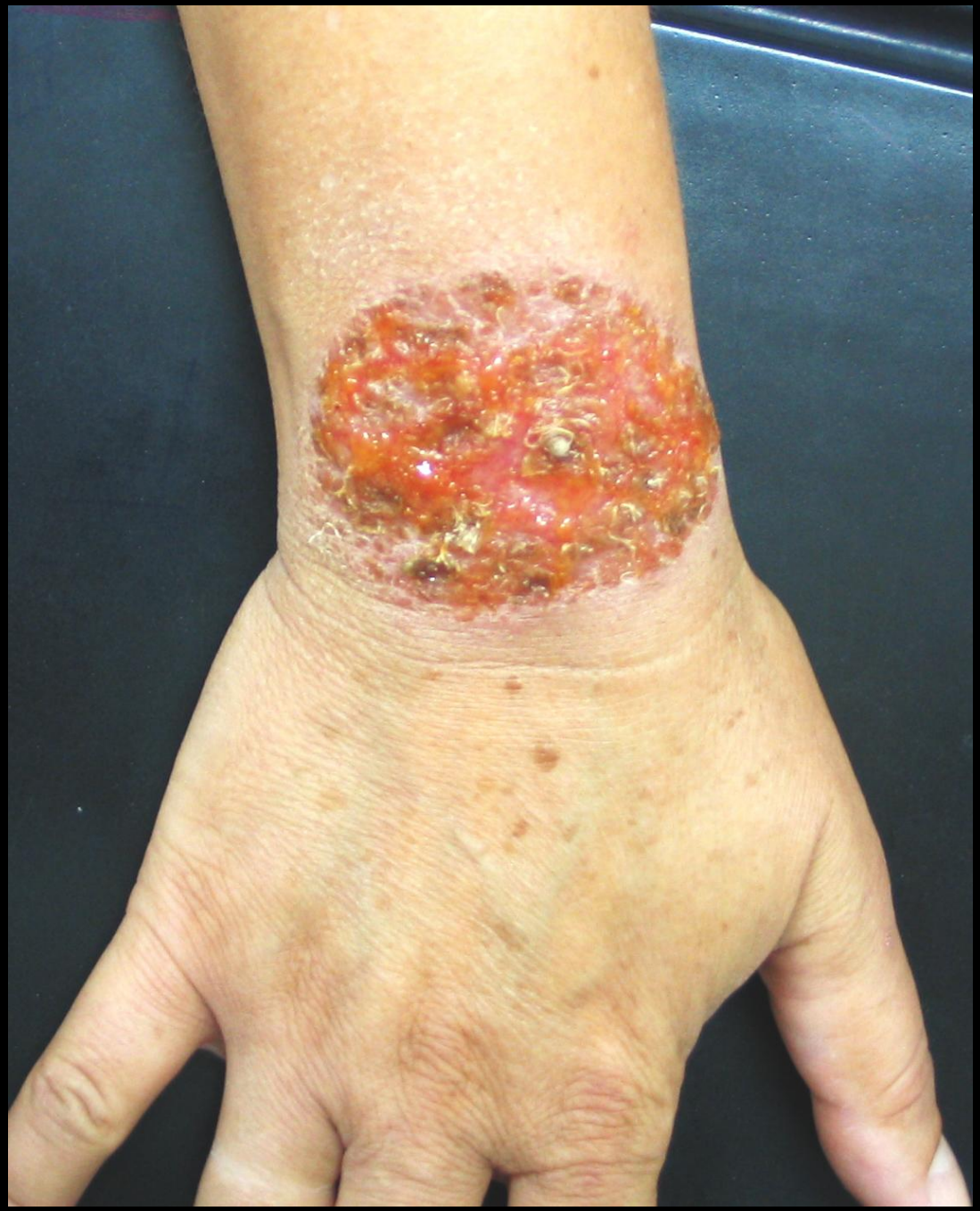


Figure from Nir-Paz et al. J Clin Microbiol 2003:5298-5301

Id reactions to dermatophytes (dermatophytids)

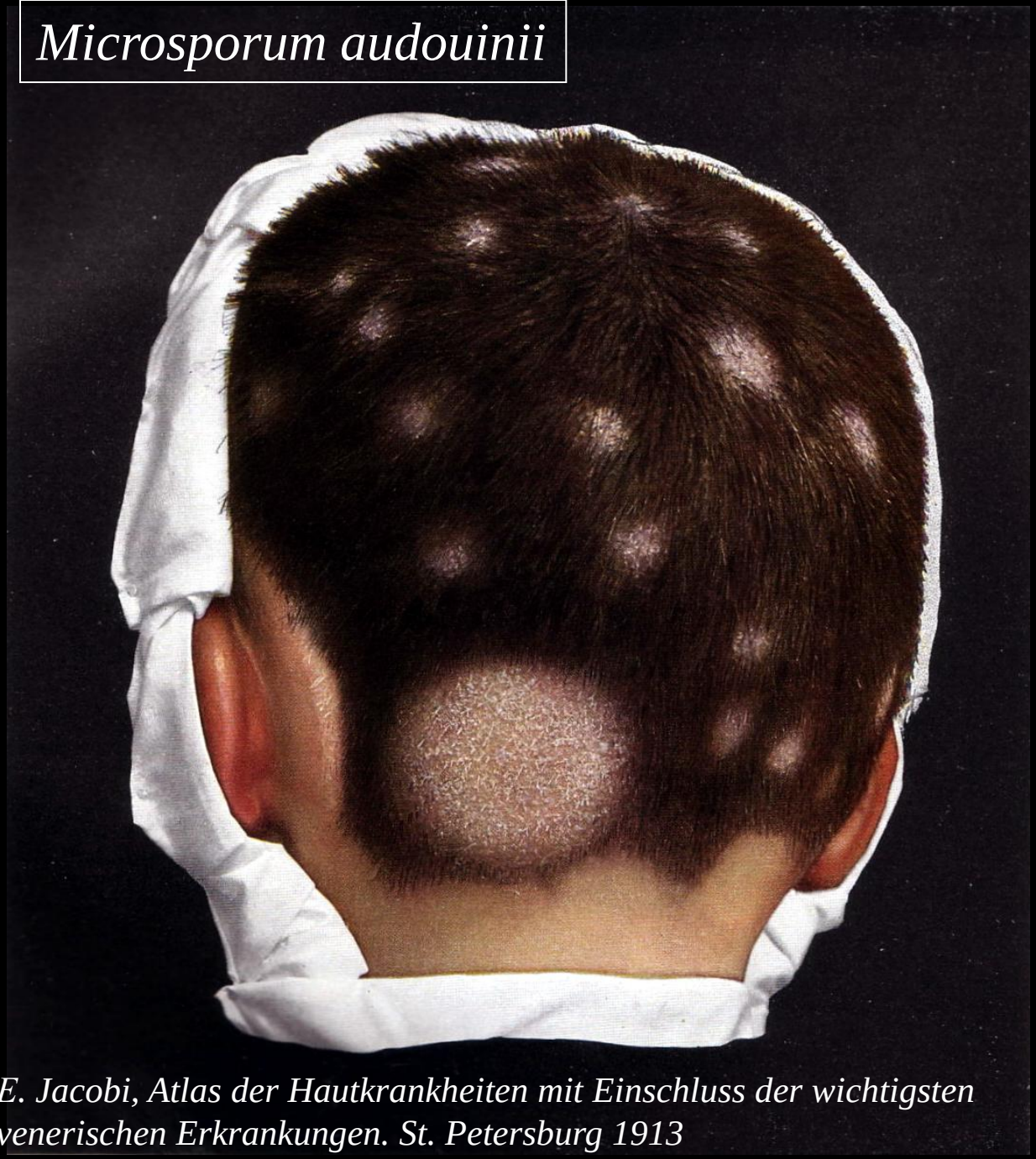




Kerion

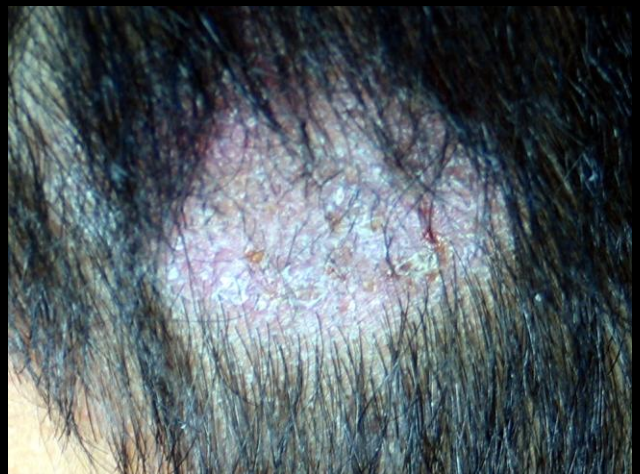
Tinea capitis

Microsporum audouinii

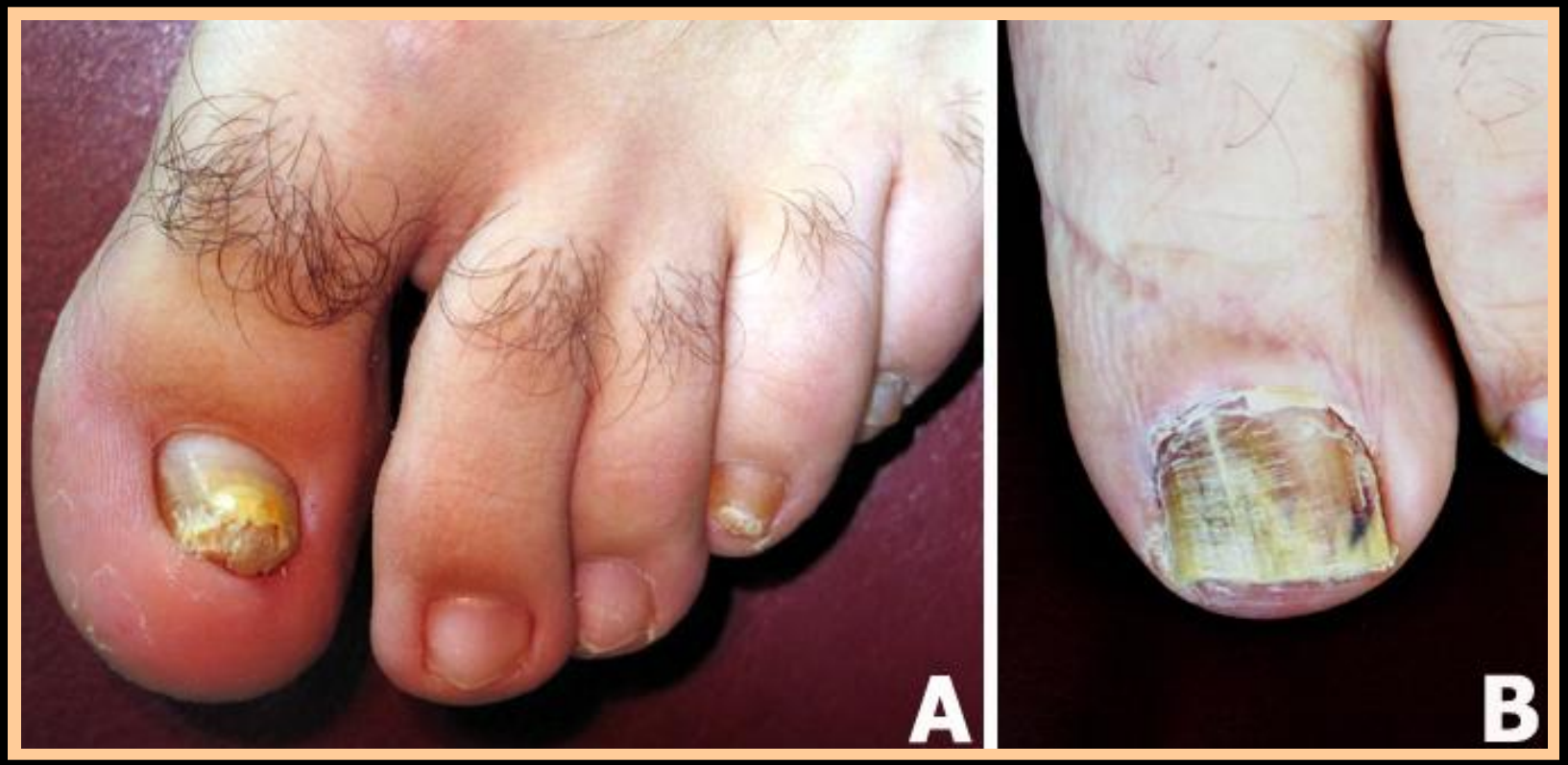


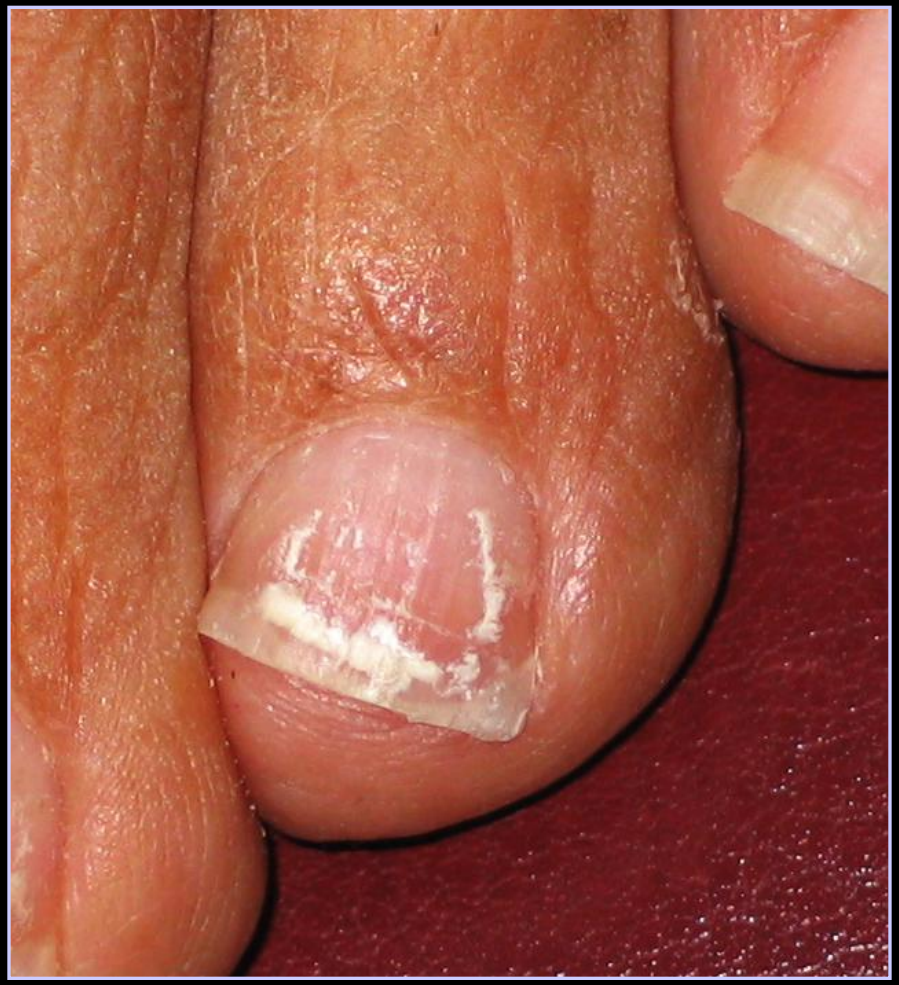
E. Jacobi, Atlas der Hautkrankheiten mit Einschluss der wichtigsten venerischen Erkrankungen. St. Petersburg 1913

Microsporum canis

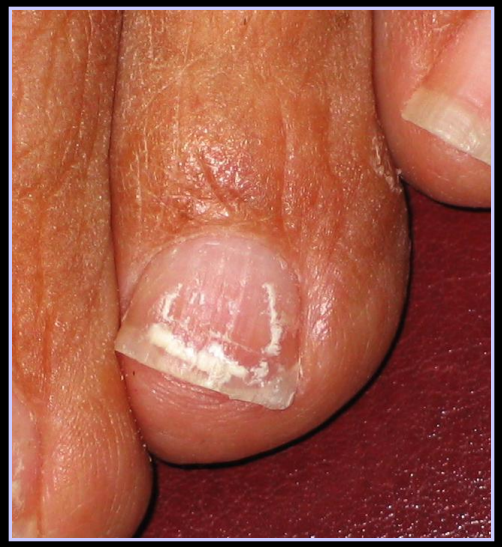


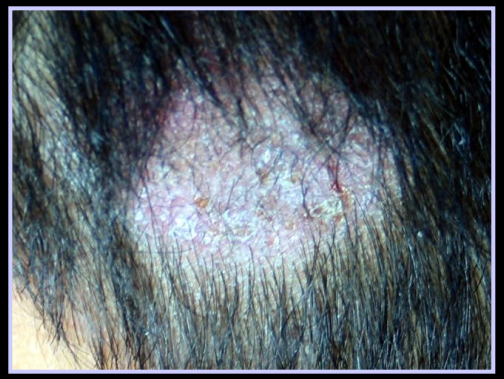
Tinea unguium





Standard diagnostic procedures for dermatophytes





Clinical examination

Laboratory investigation

Biopsy

Wood's light (tinea capitis)

Direct microscopy

Culture / Species identification

Main disadvantages of traditional dermatophyte diagnosis

- **Comparative low sensitivity**
- **Complicated identification procedure**

Procedure sequel for identification of dermatophytes in culture*

Color, topography, texture and rate of growth of colony
Microscopic morphology (macroconidia, microconidia)

↓

Slide cultures, sporulation media

↓

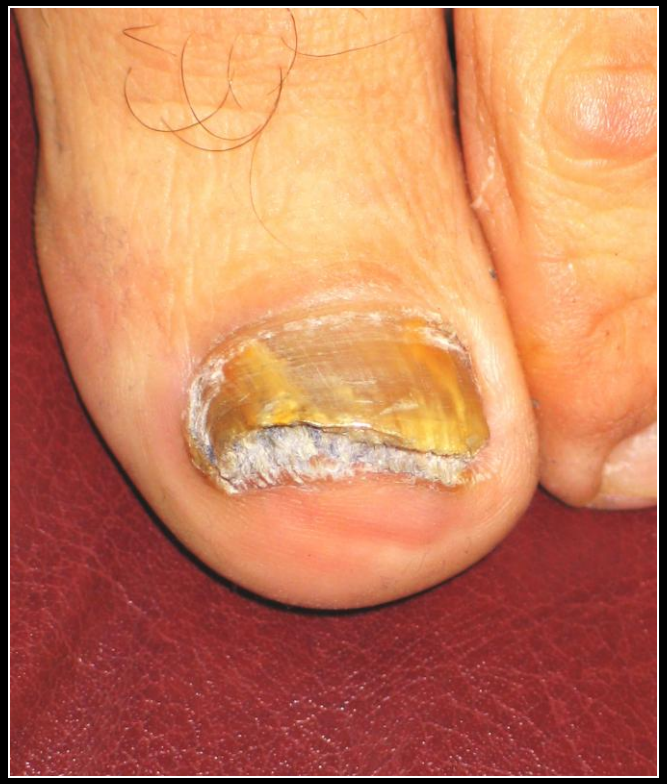
Physiological tests (nutritional requirements, rice grains, in vitro hair perforation, temperature tolerance, special differentiating media)

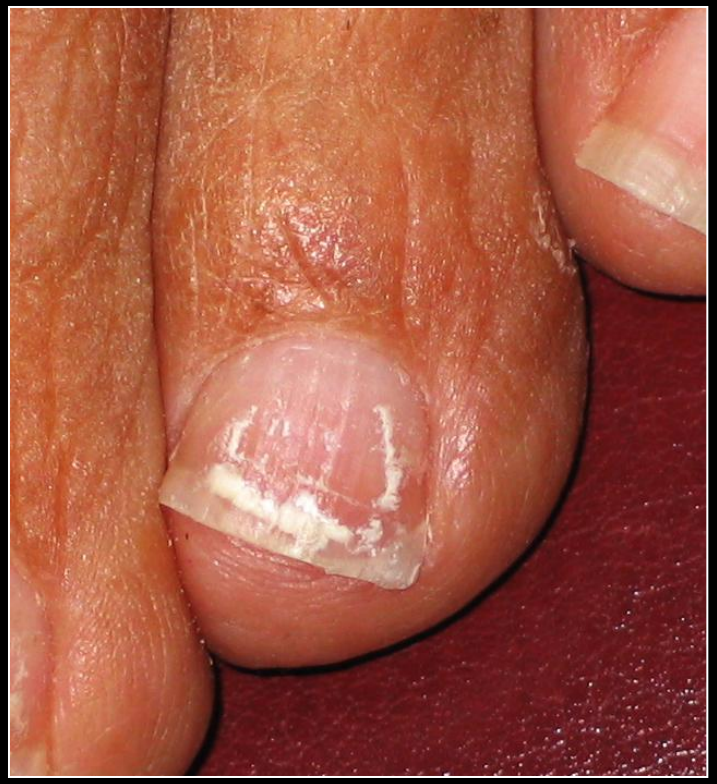
↓

Mating studies/Sequencing

**Adapted from Weitzman & Summerbell, Clin Microbiol Rev 1995:240-259*

Oychomycosis (Dermatophytes)

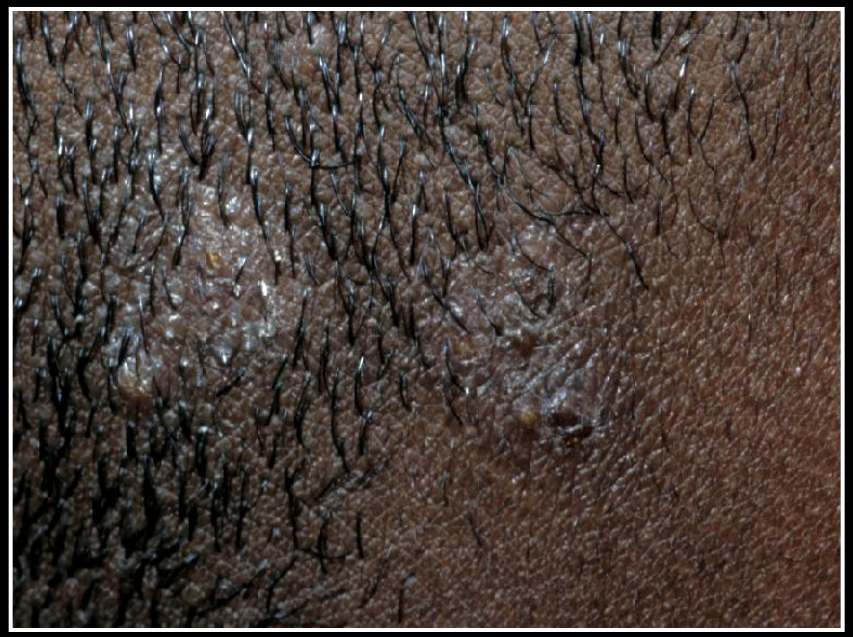


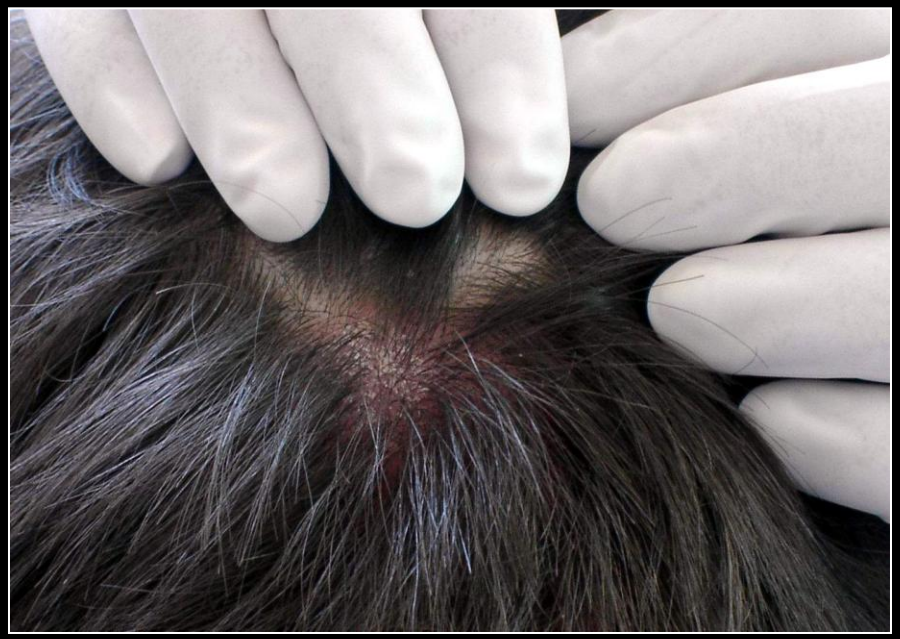


Sensitivity* (one culture attempt)	
Direct microscopy	73.8%
Culture	74.6%
Combined	83.9%

*Summerbell *et al*
Med Mycol 2005; 39-59

Tinea capitis





Sensitivity*

Direct microscopy 67.25 – 91 %

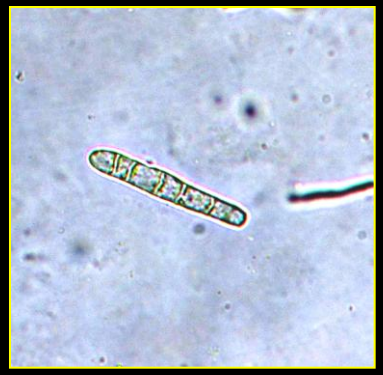
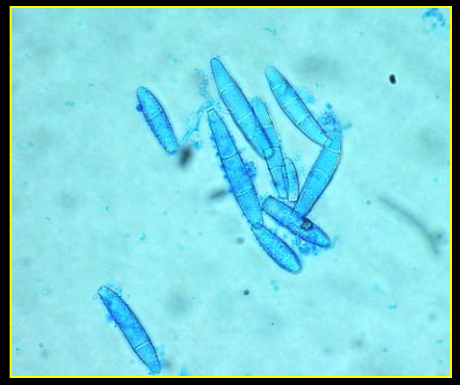
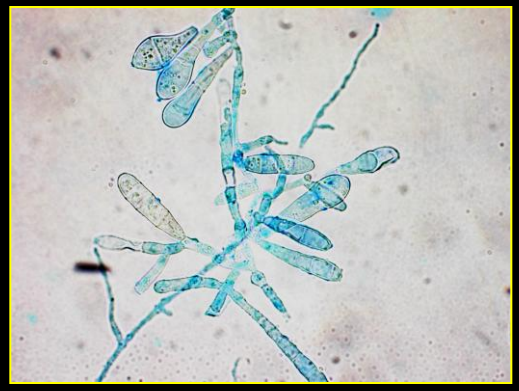
*Onsberg
Mycopathologia 1979; 153-155

Other disadvantages of traditional dermatophyte diagnosis

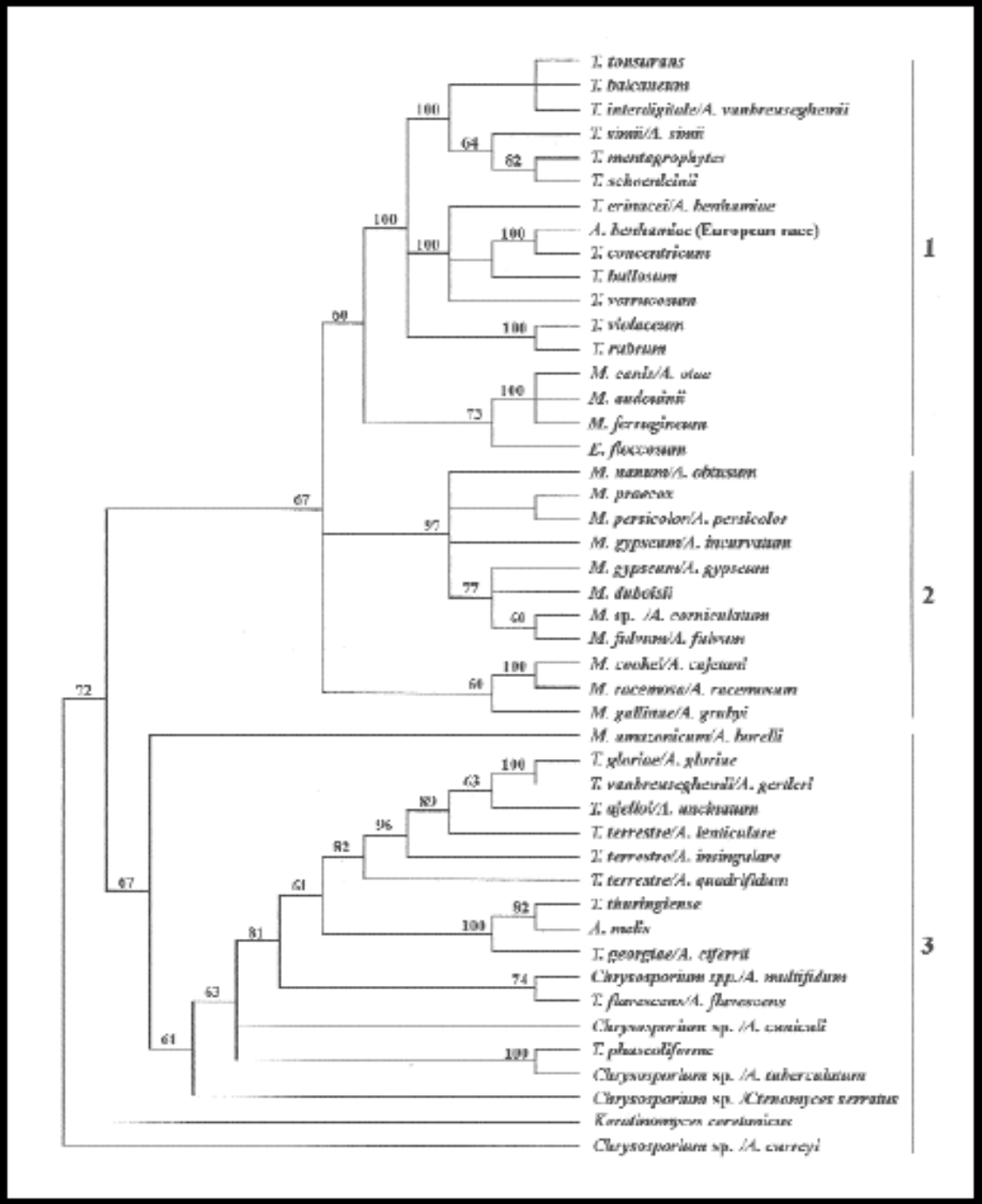
- **Slowness of culture / identification**
- **Expertise, training, experience requirements**
- **Identification mistakes**
- **The practical problem of sterile hyphae**

Anamorphic Genera of Dermatophytes

(Emmons 1934)

	Trichophyton	*Microsporum*	*Epidermophyton*
Macroconidia	Rare Cigar shaped Thin walled	Many Spindle/tapered Thick/echinulate	Many Club/blunt Thin/smooth
			
Microconidia	Numerous	Occasional Non specific	Not observed

Graser et al. Molecular Taxonomy of Dermatophytes. In Biology of dermatophytes and other keratinophilic fungi. Bilbao 2002.



Dermatophyte species*

Anthropophilic

E. floccosum
M. audouinii
M. ferrugineum
T. concentricum
*T. gourvilii**
*T. kanei**
*T. megninii**
T. mentagrophytes
*T. raubitschekii**
T. rubrum
T. schoenleinii
T. soudanense
T. tonsurans
T. violaceum
*T. yaoundei**

Zoophilic

M. canis (cat, dog)
M. equinum (horse)
M. gallinae (fowl)
M. persicolor (rodents)
T. equinum (horse)
T. mentagrophytes (rodents, rabbit, hedgehog)
T. sarkisovii (bactrian camel)
T. simii (monkeys, fowl)
T. verrucosum (cattle, sheep)

Geophilic

E. stockdaleae
M. amazonicum
A. Cookiellum anam.
M. boullardii
M. cookei
M. gypseum
M. nanum
M. praecox
M. racemosum
M. ripariae
M. vanbreuseghemii
T. ajelloi
T. flavescens
T. gloriae
*T. longifusum**
T. phaseoliforme
T. terrestre
T. vanbreuseghemii

*Adapted from Weitzman & Summerbell, Clin Microbiol Rev 1995:240-259

Not regarded as independent species

Molecular techniques for dermatophytes I

- Species identification from pure culture

PCR-RFLP

RAPD

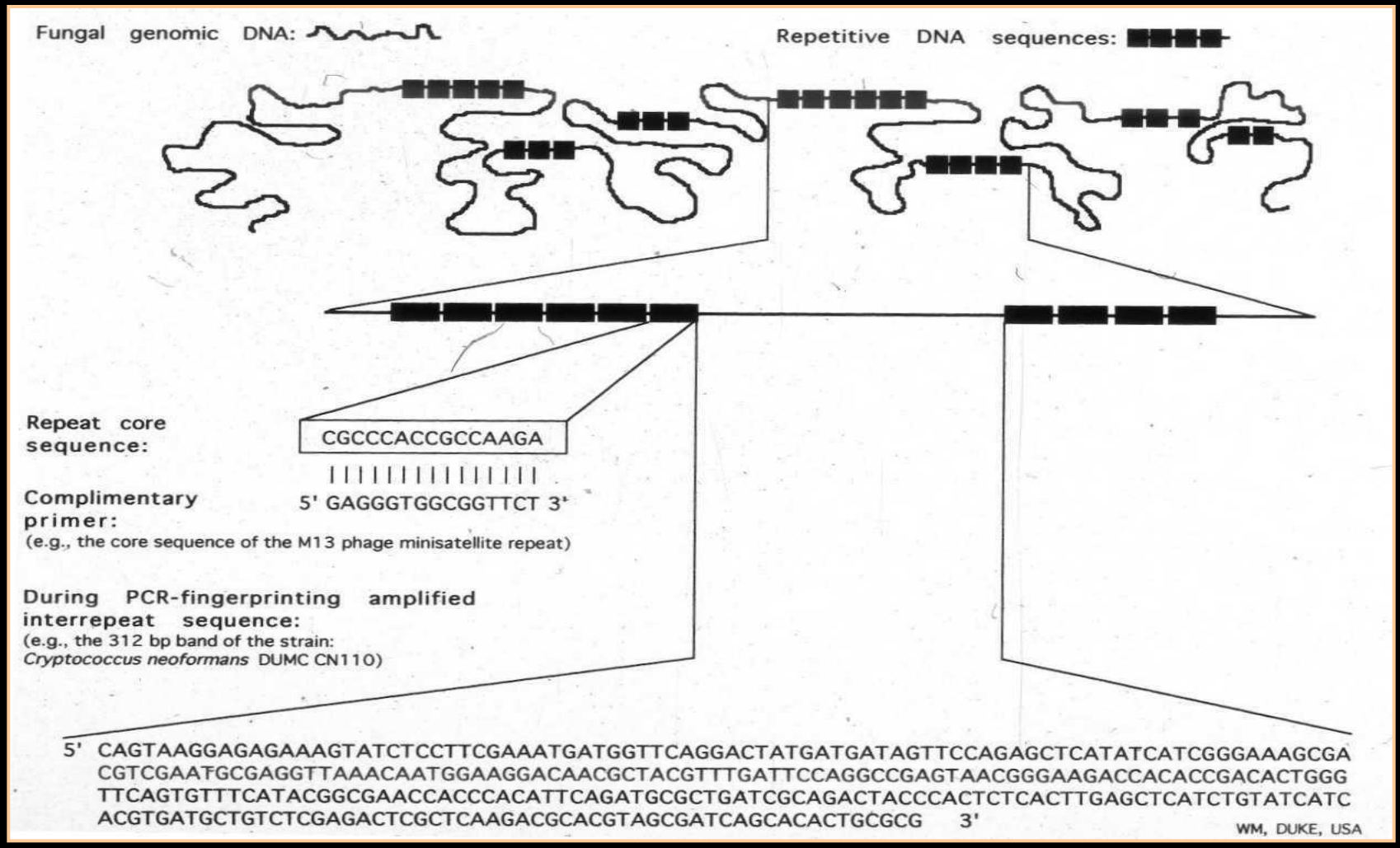
PCR-fingerprinting

Sequencing (as per Sanger)

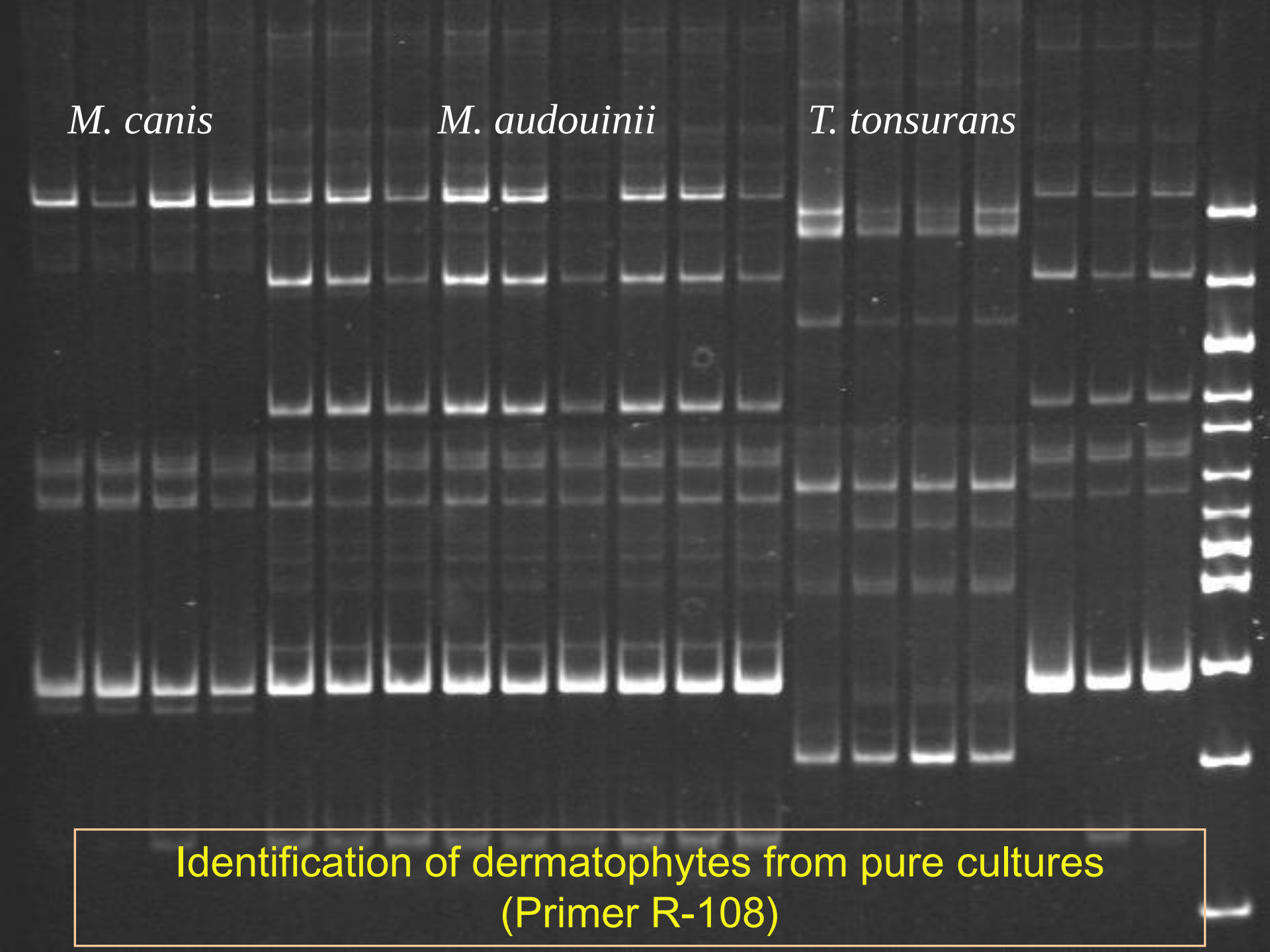
Other methods

Molecular identification of dermatophytes *in vitro*

- Standard identification of dermatophytes is based on morphological, physiological and biochemical characters
- Various molecular methods have been sporadically used as an aid to identification (PCR-RFLP, DNA-fingerprinting)
- The current standard molecular method for dermatophyte identification is ITS sequencing
- Sequencing has yet to be standardised for clinical use
- "DNA Barcoding" of dermatophytes has started the stardardisation process *Summerbell et al. Med Mycol; 2007.*



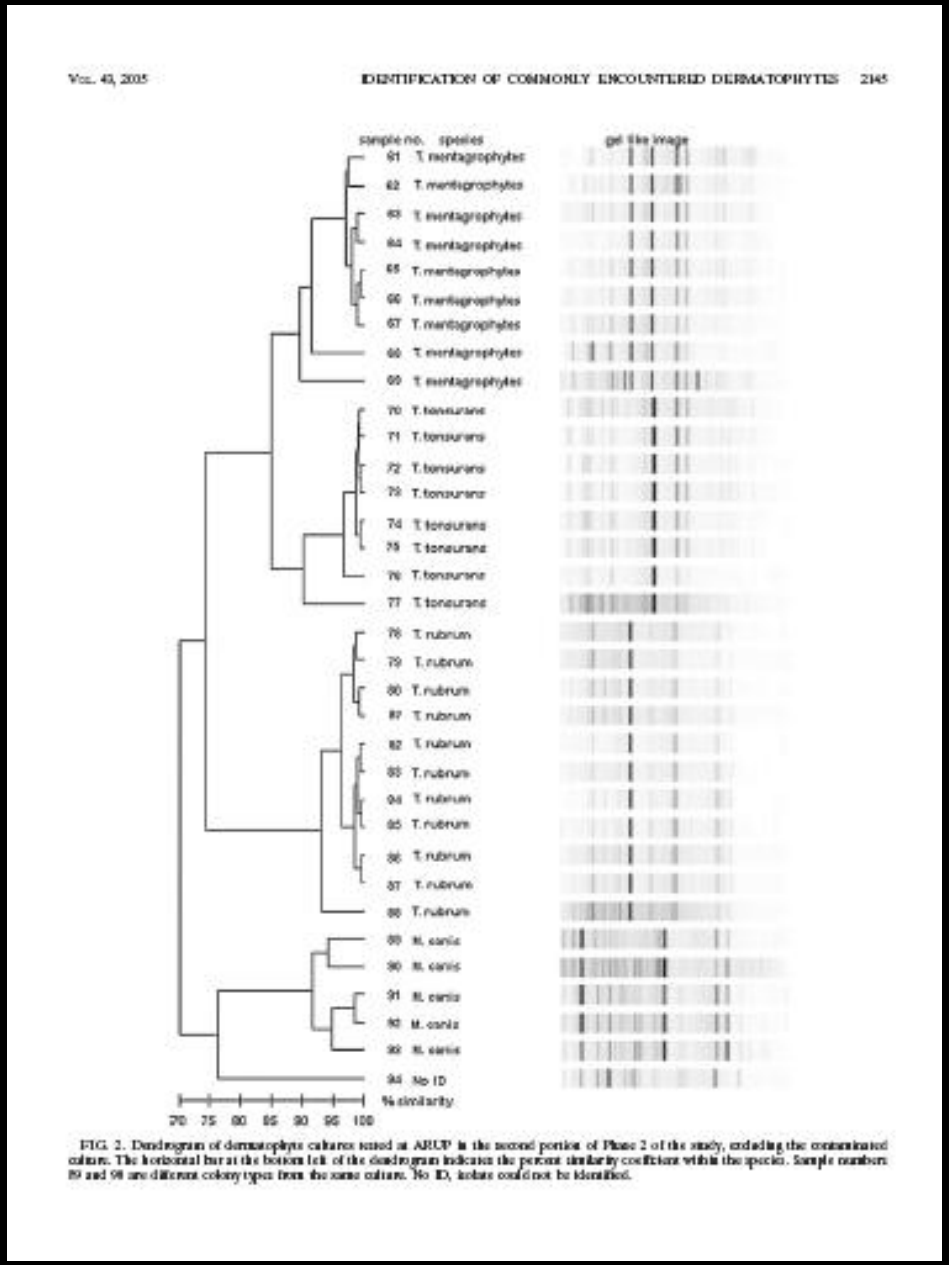
PCR-fingerprinting amplifies hyper-changeable areas between the repeat element (Figure by Wieland Meyer, Univ. of Sydney, Australia)



Identification of dermatophytes from pure cultures
(Primer R-108)

Indicative ribotyping of different dermatophyte species, based on repeat elements of the IGS rDNA region
The method can be used for dermatophyte species identification

Diversilab System



VOL. 43, 2005 IDENTIFICATION OF COMMONLY ENCOUNTERED DERMATOPHYTES 2145

sample no. species gel like image

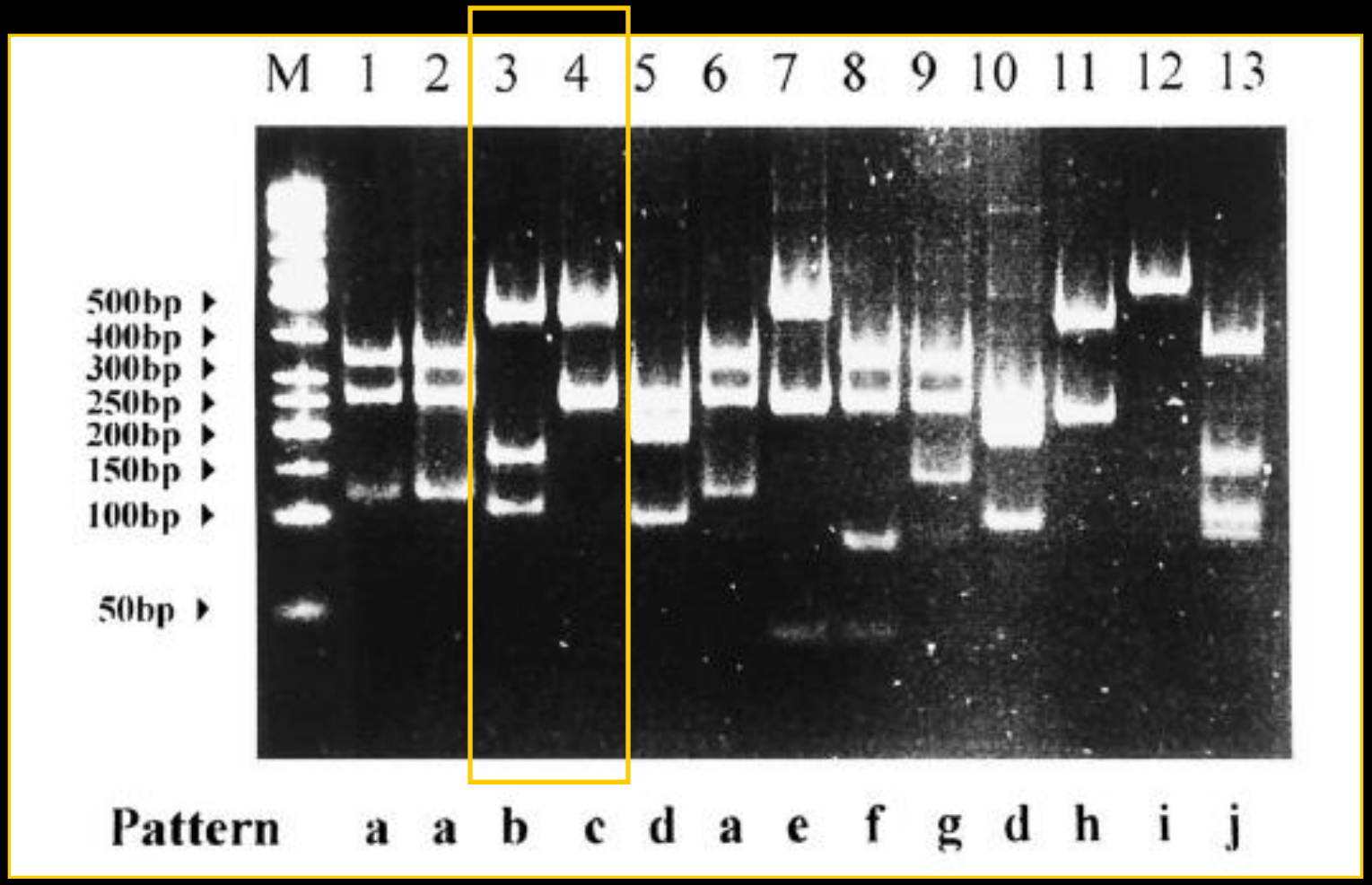
61 T. mentagrophytes
62 T. mentagrophytes
63 T. mentagrophytes
64 T. mentagrophytes
65 T. mentagrophytes
66 T. mentagrophytes
67 T. mentagrophytes
68 T. mentagrophytes
69 T. mentagrophytes
70 T. tonsurans
71 T. tonsurans
72 T. tonsurans
73 T. tonsurans
74 T. tonsurans
75 T. tonsurans
76 T. tonsurans
77 T. tonsurans
78 T. rubrum
79 T. rubrum
80 T. rubrum
81 T. rubrum
82 T. rubrum
83 T. rubrum
84 T. rubrum
85 T. rubrum
86 T. rubrum
87 T. rubrum
88 T. rubrum
89 M. canis
90 M. canis
91 M. canis
92 M. canis
93 M. canis
94 No ID

% similarity
70 75 80 85 90 95 100

FIG. 2. Dendrogram of dermatophyte cultures tested at ARUP in the second portion of Phase 2 of the study, excluding the contaminated cultures. The horizontal bar at the bottom left of the dendrogram indicates the percent similarity coefficient within the species. Sample numbers 89 and 90 are different colony types from the same culture. No ID, isolate could not be identified.

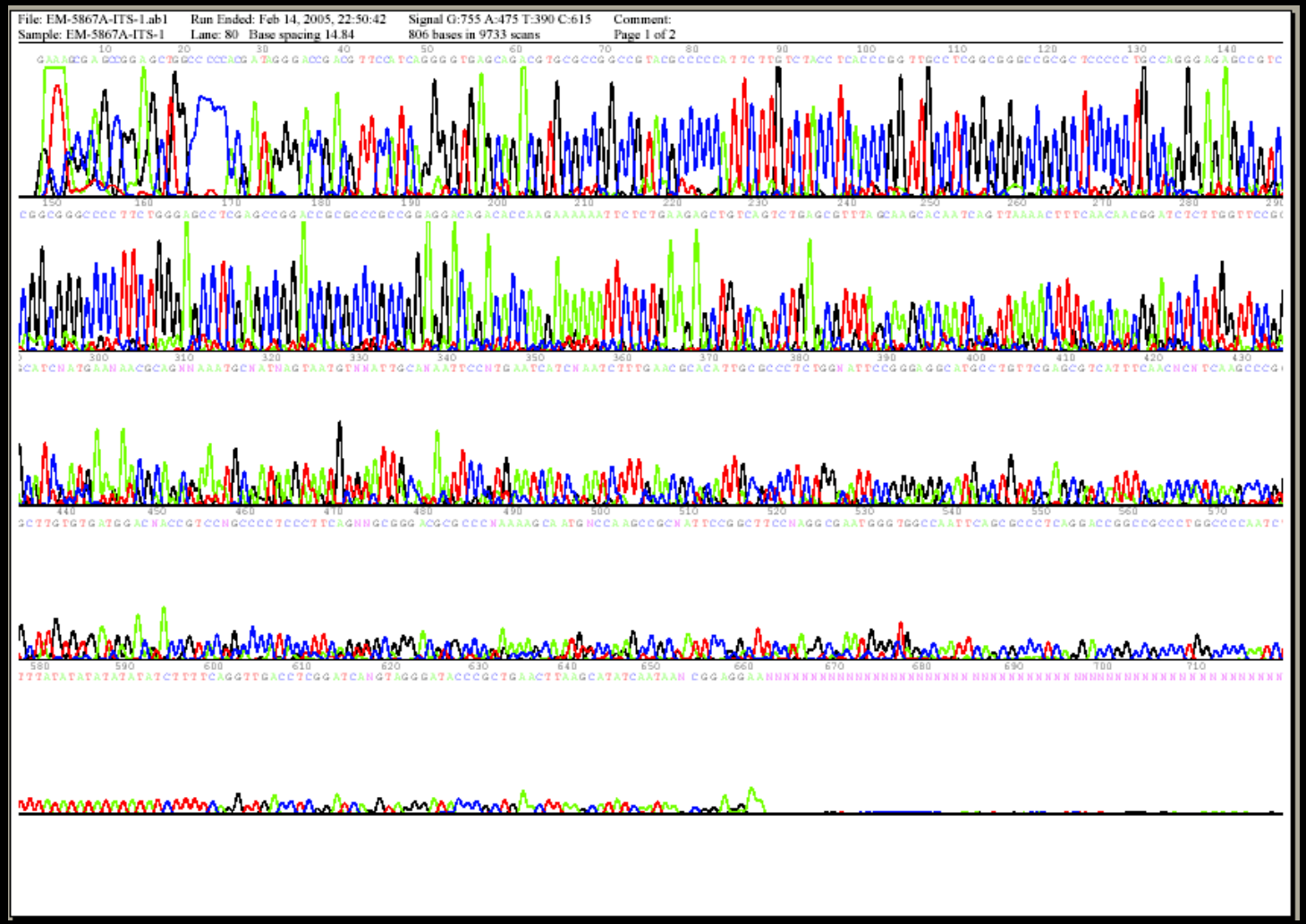
Pounder et al, JCM 2005:2141-2147

Trichophyton mentagrophytes



PCR - RFLP patterns (ITS x *Dde*I)

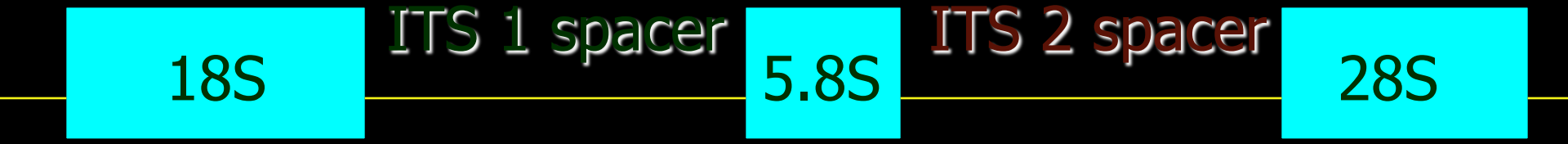
Shin et al. JAAD, 2003, 6: 857-865

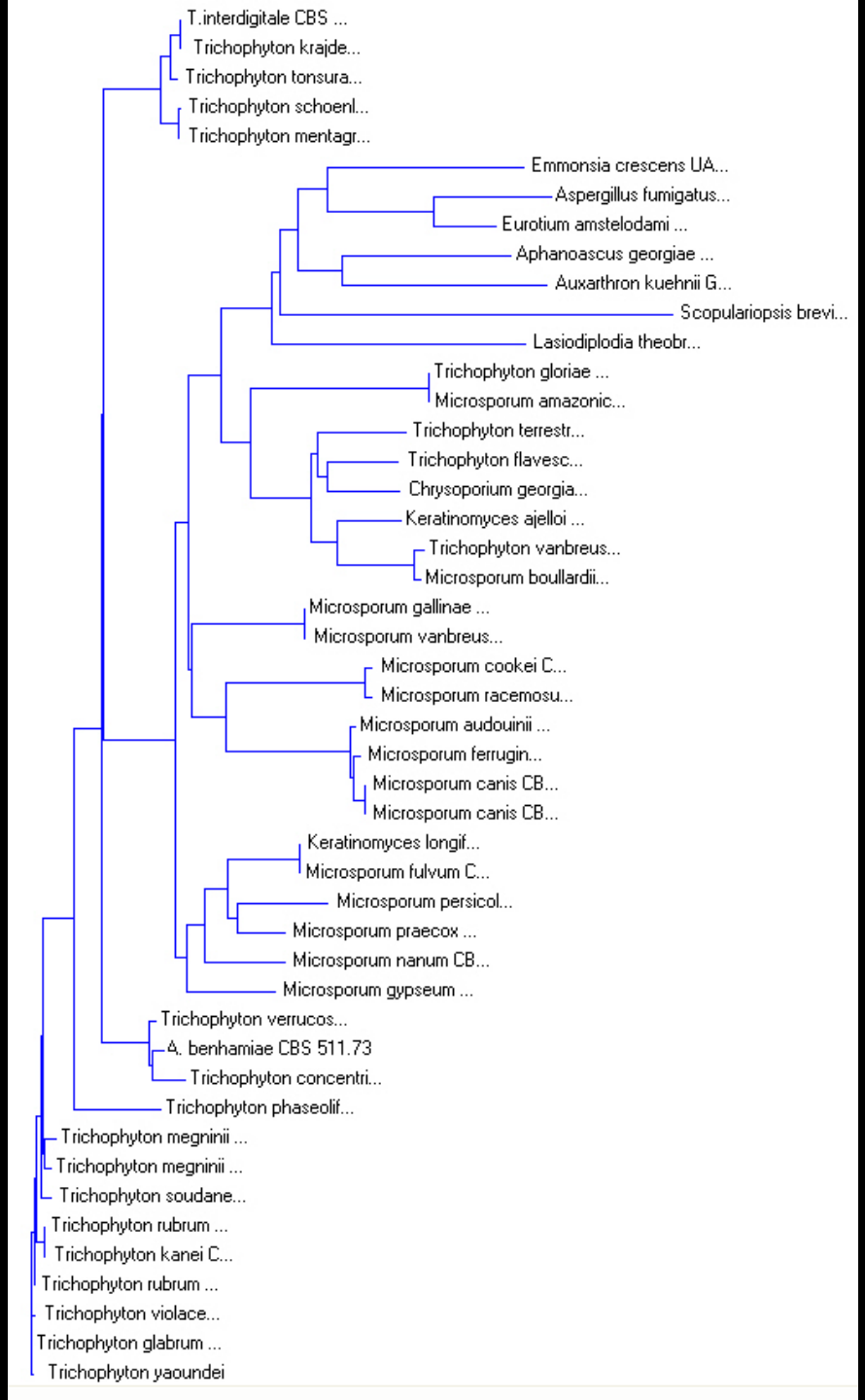


Sequencing for dermatophyte identification

ITS or 28S rRNA

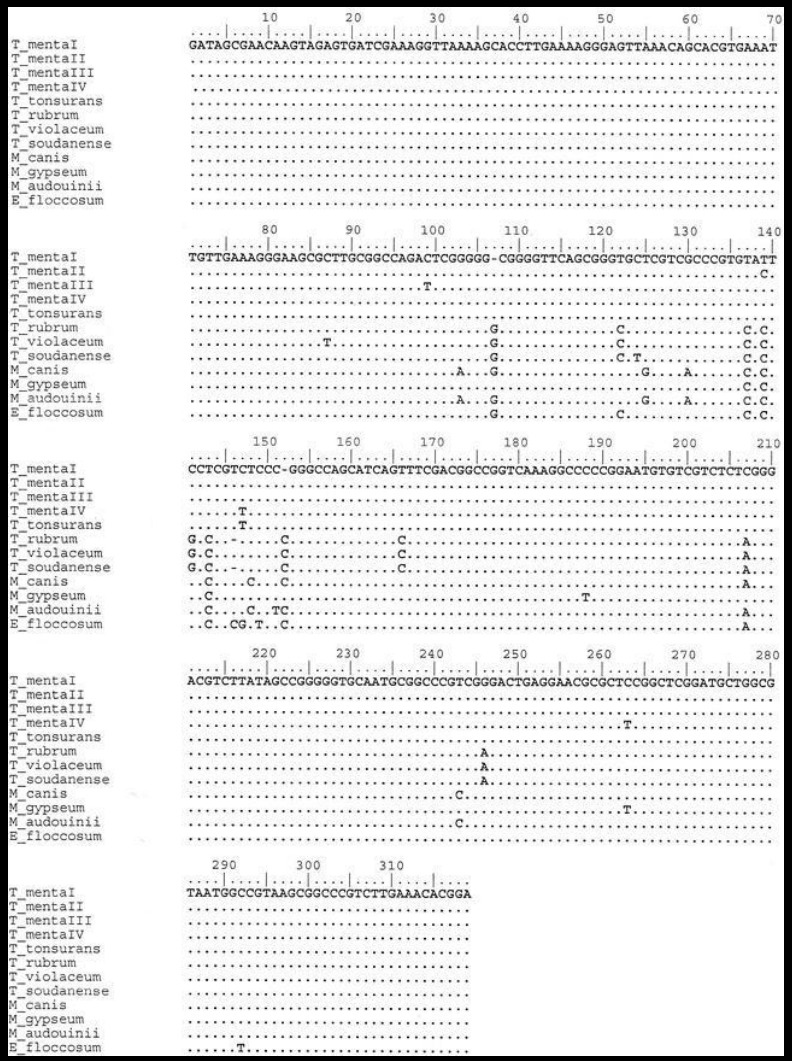
Multicopy ribosomal DNA region





Dendrogram exemplifying distinct dermatophyte clusters based on ITS sequences

28S ribosomal gene



MicroSeq D2 LSU rRNA Fungal Sequencing Kit

Ninet et al, JCM 2003, 41:826-830

Molecular techniques for dermatophytes II

- Detection and identification of the species, directly from clinical material

PCR-RFLP

PCR

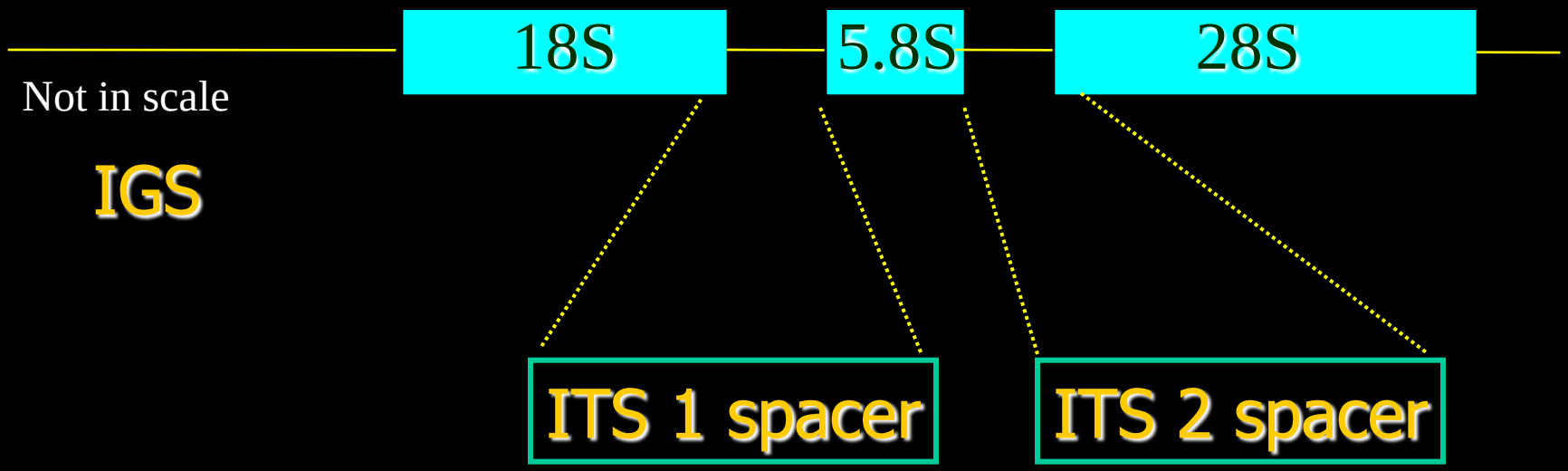
Real-time PCR

PCR-ELISA

DNA Targets

for Species-Specific Dermatophyte Diagnostic Assays

- **Internal Transcribed Spacer**
- **Intergenic Spacer**
- **Chitin synthase 1**
- **Microsatellite DNA**
- **Other**

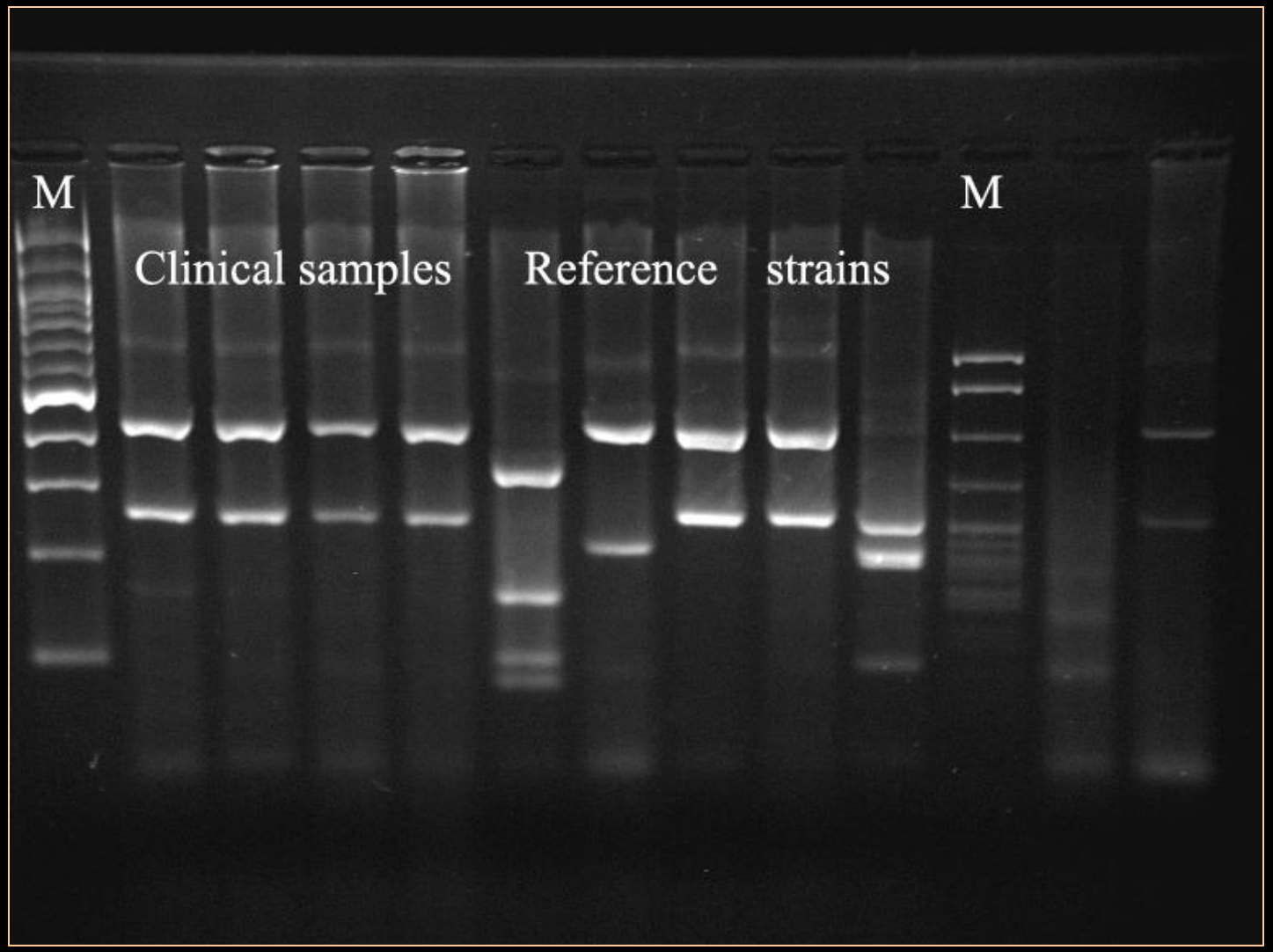


PCR for Detection of Dermatophytes I

General Fungal Primers

- 2000 Pan-fungal 18s assay, highly sensitive but unspecific
  Turin et al. Eur J Clin Invest 2000; 30:511-508.
- 2003 Dermatophyte specific (?) chitin synthase 1 primers, unspecific
  Kano et al. J Vet Med Sci 2003; 64:267-270.
- 2004 First real-time PCR–RFLP assay, a number of general fungal primers tested, unspecific
  Gutzmer et al. J Med Microbiol 2004; 53: 1207–14.
- 2007 Pan-dermatophyte PCR in onychomycosis, unspecific
  Garg et al. J Clin Microbiol 2007; 45: 3443–3445.

PCR RFLP



PCR - RFLP patterns (ITS x *Dde*I), derived from rabbit clinical material

Arabatzis et al. Vet Dermatol 2006:322-326

PCR-RFLP

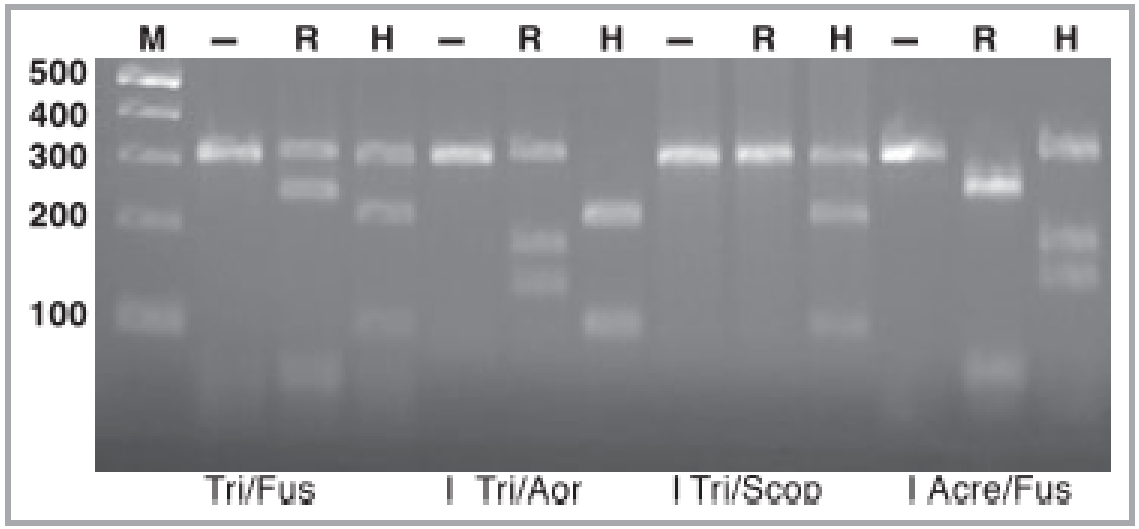


Fig 2. Identification of mixed infections in four nails using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. *Trichophyton rubrum*, *Aspergillus oryzae*, *Scopulariopsis brevicaulis* and *Fusarium* spp. grew as a single species in culture from nails 1, 2, 3 and 4, respectively. RFLPs from nails 1, 2, 3 and 4 are compatible, respectively, with the presence of *Trichophyton* spp. and *Fusarium* spp. or *Candida* spp. (Tri/Fus), *A. oryzae* and *Trichophyton* spp. (Tri/Aor), *S. brevicaulis* and *Trichophyton* spp. (Tri/Scop), and *Fusarium* spp. and *Acremonium* spp. (Acre/Fus). M, 100 bp ladder; –, undigested DNA; R, *Rsa*I digestion; H, *Hinf*I digestion.

Bontems et al. BJD 2009:791-796

Dermatophyte Detection PCR Assays II
Species distinction is based on specific primers

- *Trichophyton rubrum* assay plus a *Scopulariopsis brevicaulis* assay, (T1 microsatellite target)

  Kardjeva V et al. J Clin Microbiol 2006; 44:1419-1427.

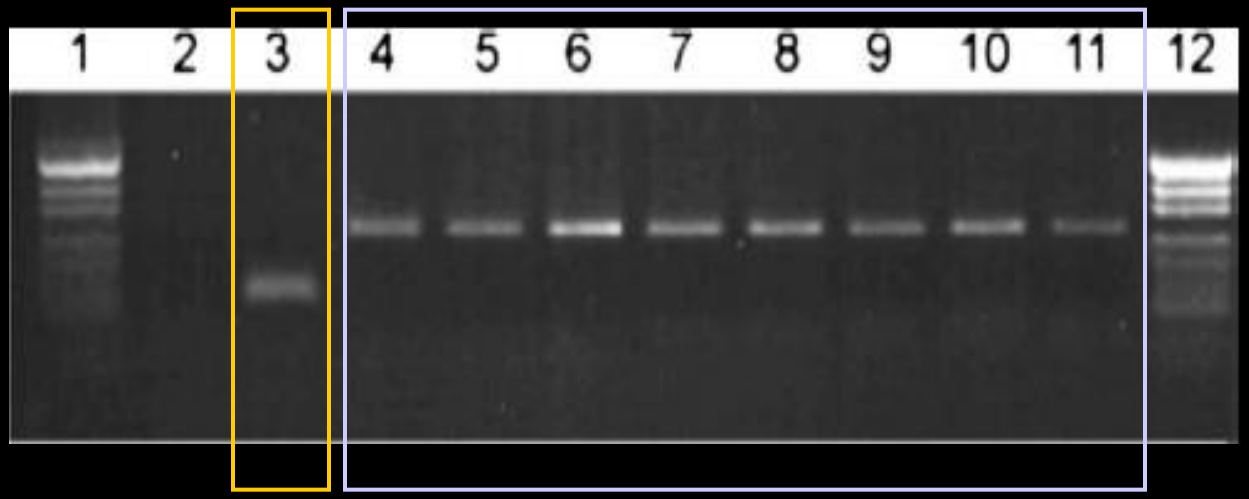
- *T. rubrum* detection assay (ITS based), multiplexed with a pan-dermatophyte assay (chitin synthase 1)

  Brillowska-Dabrowska A et al. J Clin Microbiol 2007; 45: 1200-1204.

- *Microsporum canis* and *M. audouinii* detection assay (microsatellite DNA)

  Roque VD et al. J Clin Microbiol; 2006.

Trichophyton rubrum specific PCR



General-dermatophyte PCR

Dermatophytes multiplex PCR ® kit, Statens Institute

Brillowska-Dabrowska A et al. J Clin Microbiol; 45: 1200-1204.

Dermatophyte Detection PCR Assays II

Real-time PCR: species distinction is based on specific probes

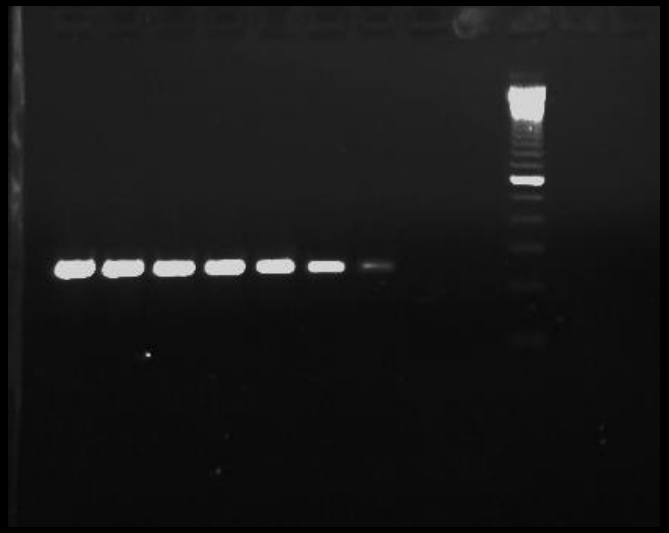
- *Trichophyton tonsurans*, (IGS based)

  Sugita et al. Med Mycol 2006; 44:579-581.

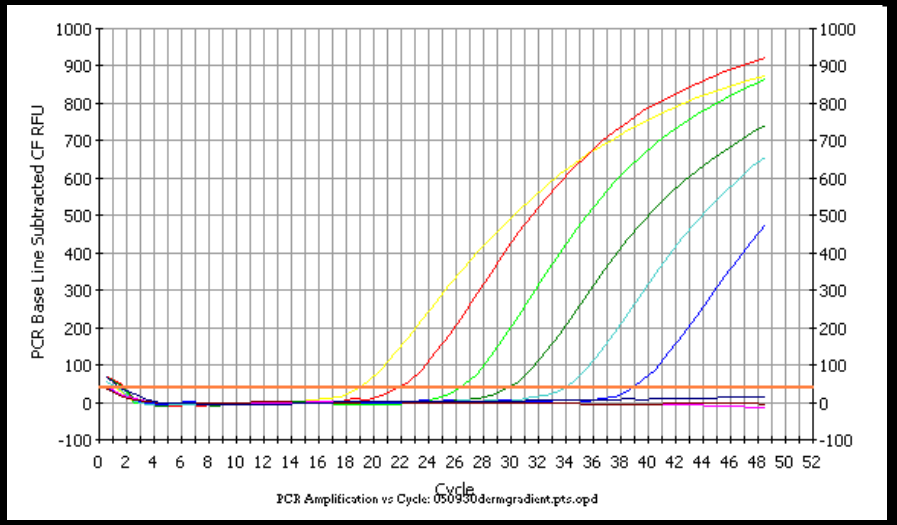
- *T. rubrum / T. mentagrophytes / T. violaceum / T. tonsurans / M. canis / M. audouinii,* two multiplex assays using specific probes and general-dermatophyte primers (ITS based)

  Arabatzis et al. Brit J Dermatol 2007.

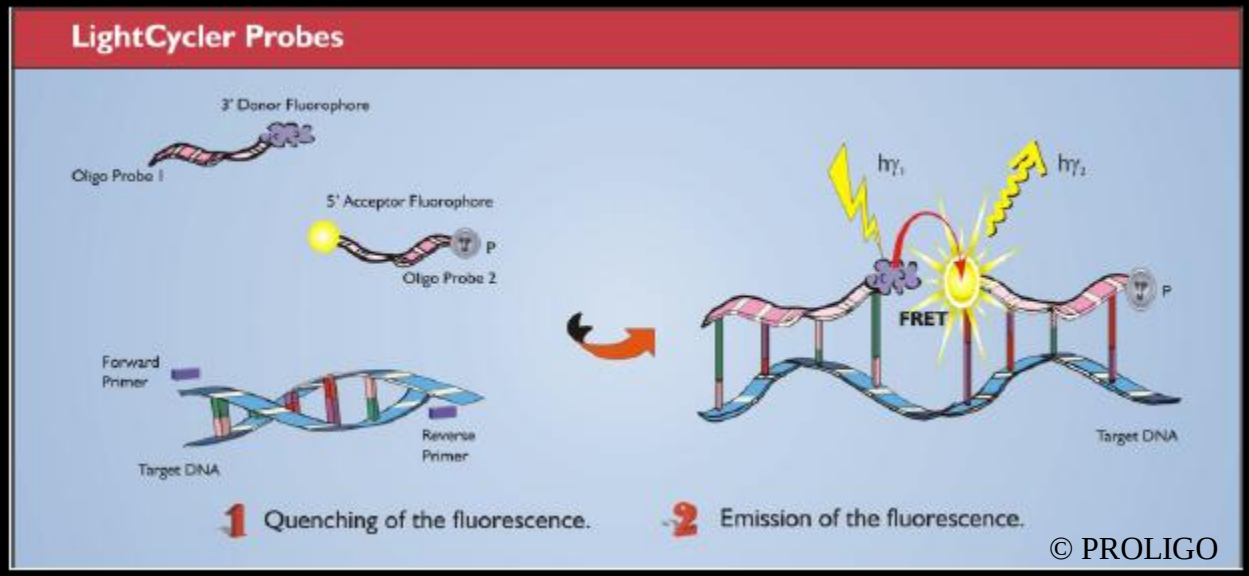
Conventional PCR PCR



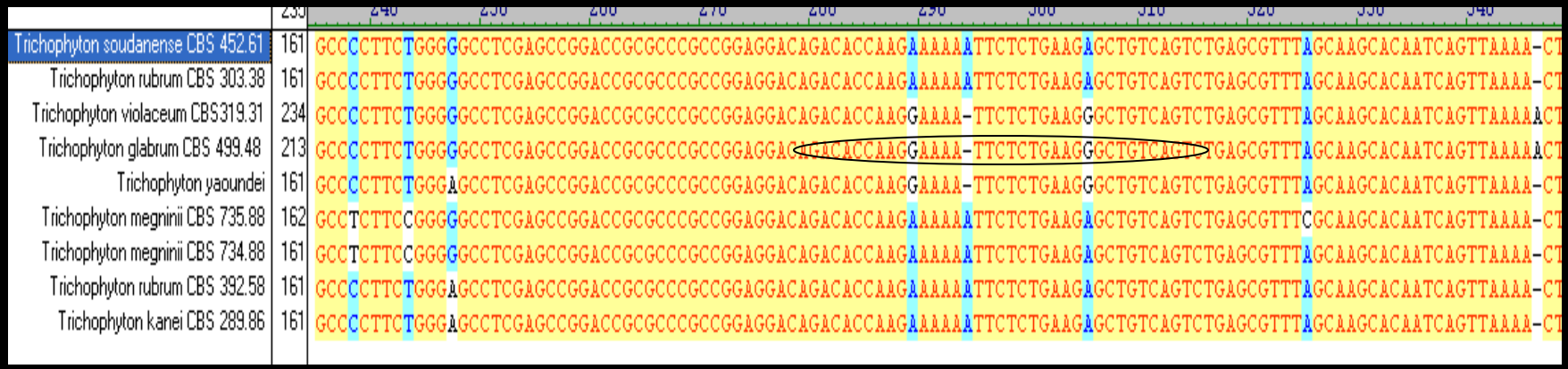
Real-time PCR



Principle of real-time PCR



An example of species-specific probe: *T. violaceum*



Arabatzis et al. Brit J Dermatol 2007.

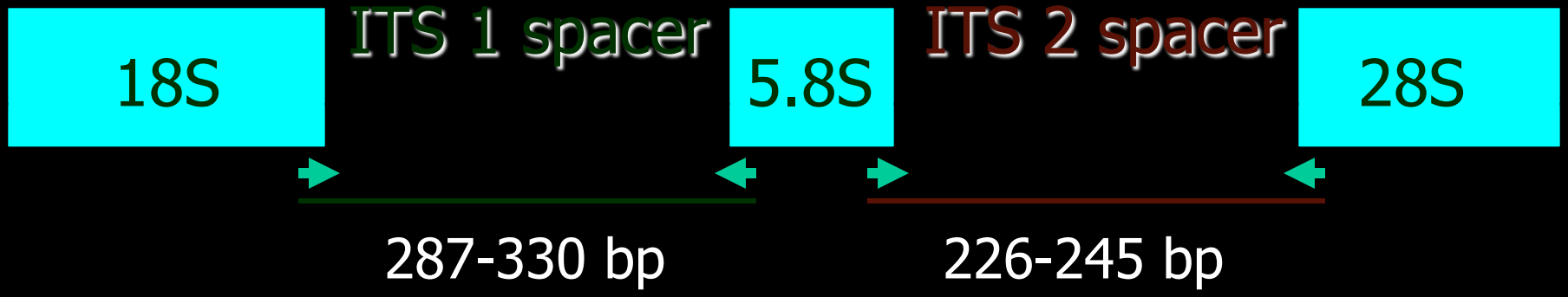
Final design

Multiplex I

T. mentagrophytes species complex MGB
T. violaceum Taqman
T. tonsurans MGB

Multiplex II

T. rubrum species complex Taqman
M. canis MGB
M. audouinii MGB



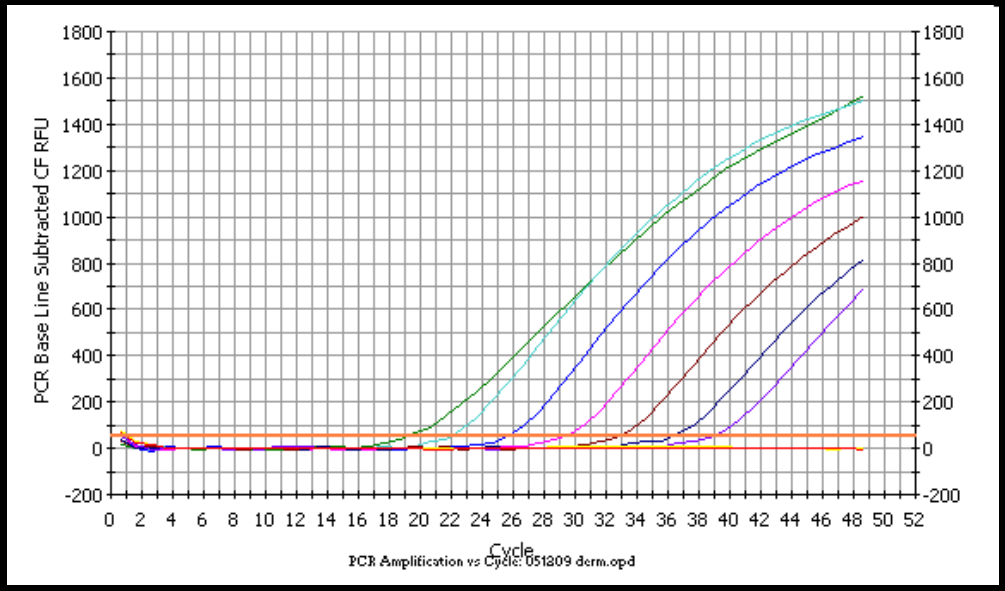
Reaction III-Internal control

Phocid Herpes Virus 1

Arabatzis et al. Brit J Dermatol 2007.

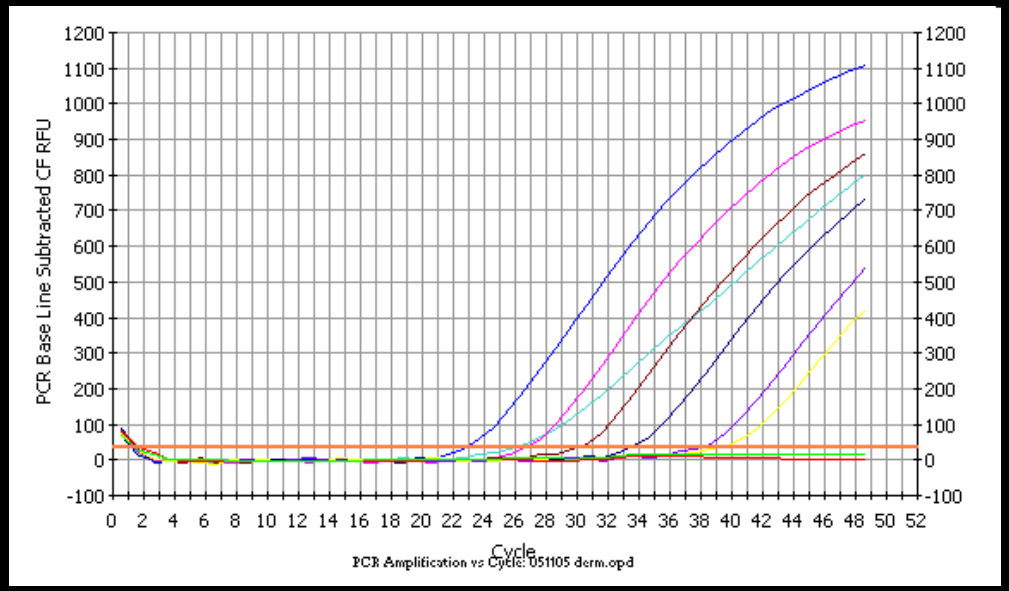
Sensitivity

Multiplex: *M. audouinii*



DNA serial dilutions
Sensitivity: 100 fg

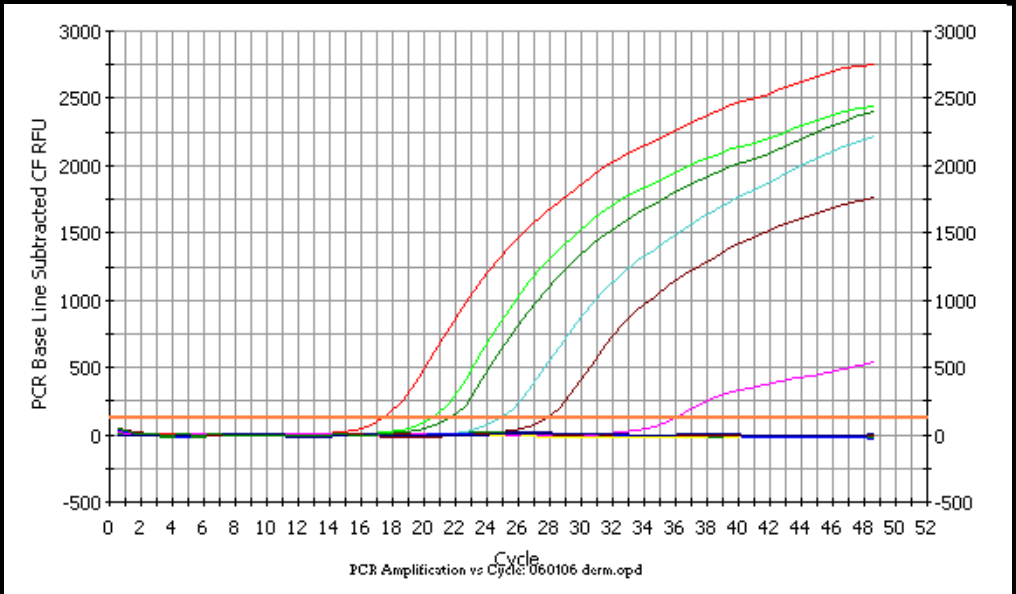
Simplex: *M. audouinii*



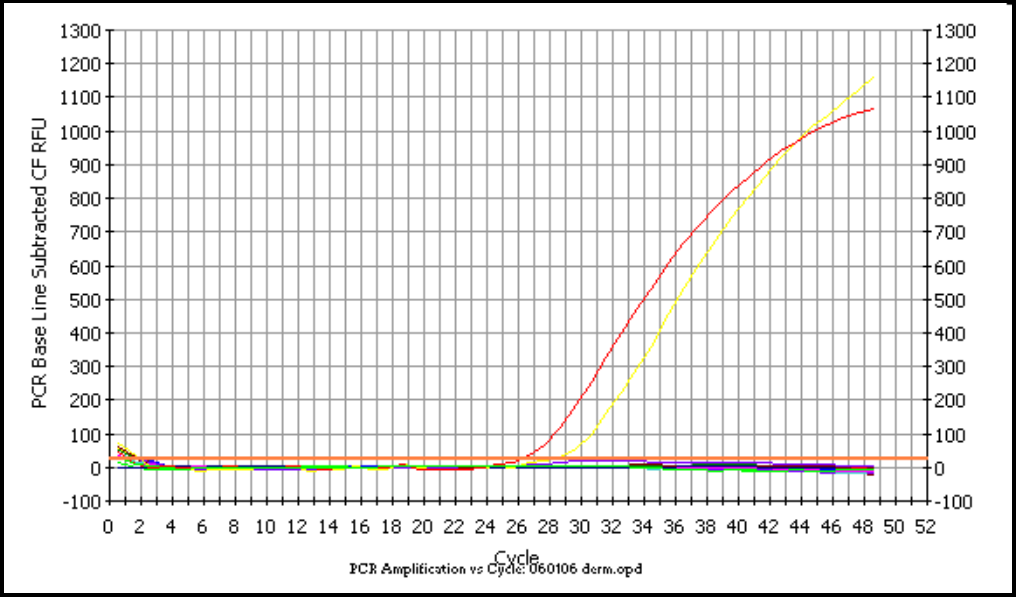
Arabatzis et al. Brit J Dermatol 2007.

Results: Clinical Specimens

Specimens positive for *T. rubrum*



Specimens positive for *M. audouinii*



Arabatzis et al. Brit J Dermatol 2007.

Results

92 specimens from cases suspicious for dermatophytes

Standard methods	+	40
Culture +/Microscopy	+	29
Culture -/Microscopy	+	11
PCR	+	47
PCR -/Microscopy nails, *Aspergillus*	+	1
Double infections (*T. rubrum* & *T. mentagrophytes*)	+	2

Arabatzis et al. Brit J Dermatol 2007.

Dermatophyte Detection PCR Assays III

Species distinction is based on hybridization of PCR products to specific probes

ARRAY

- Seventeen species ITS based, not tested with clinical samples

Li HC et al. J Clin Microbiol 2007; 45: 3160-3166.

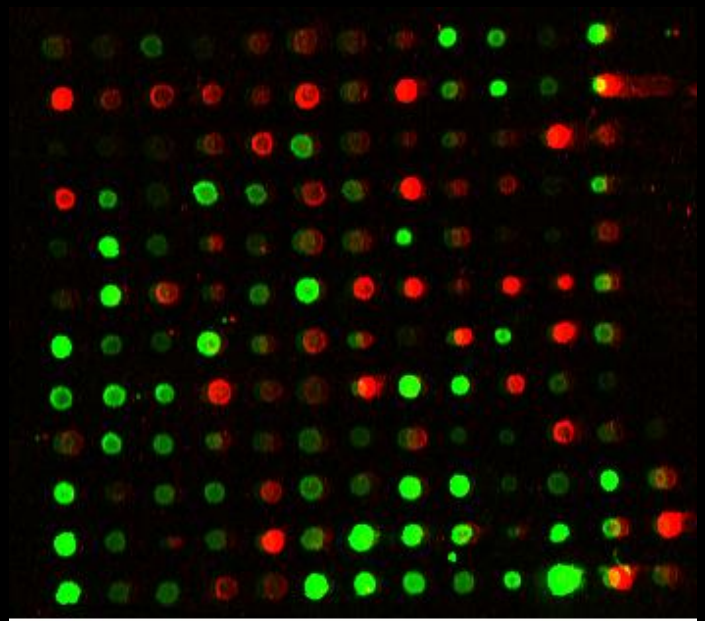
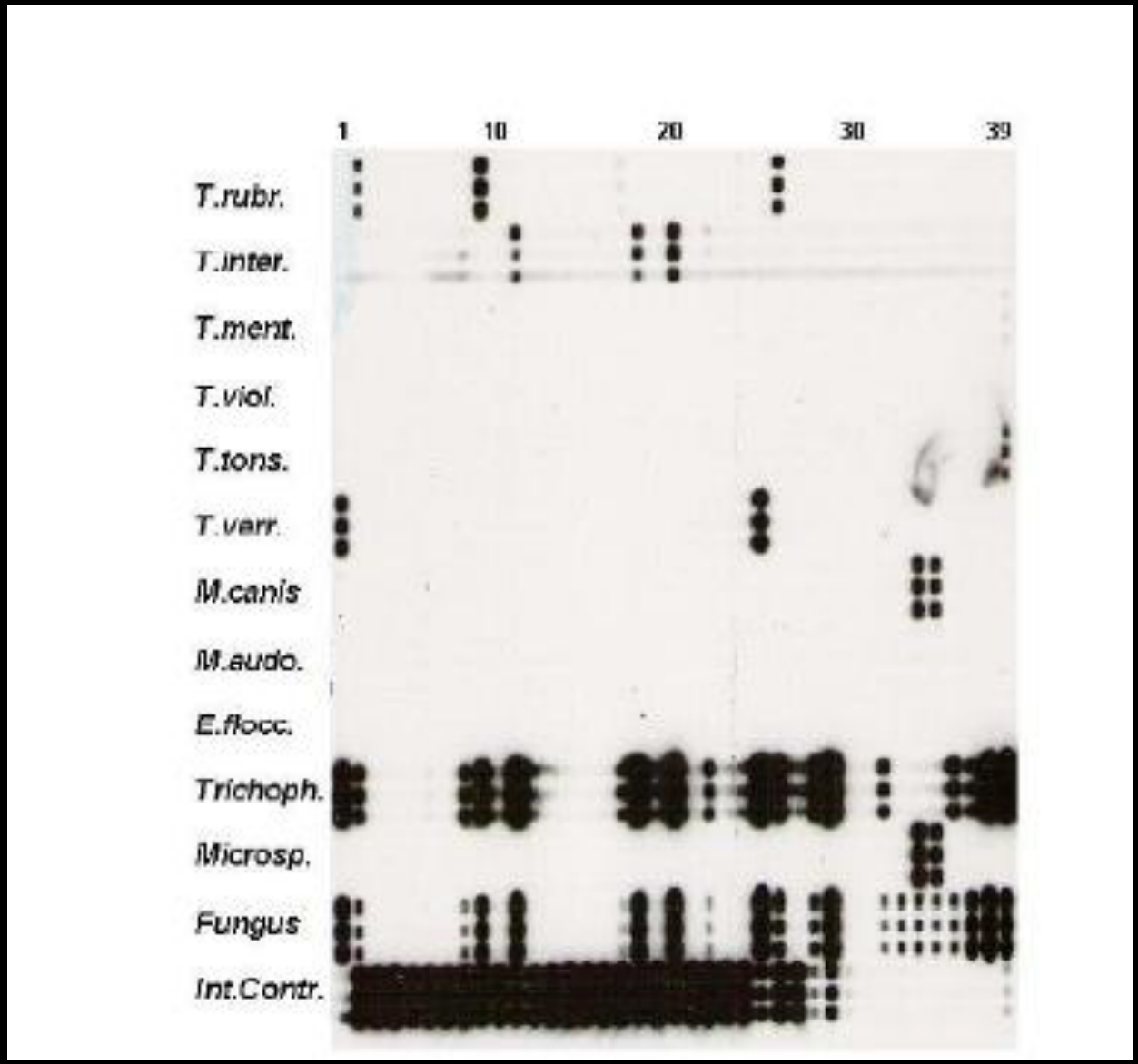


Figure from A. Velegraki & E. M. Kambouris, Archives of Greek Medicine, 2003, 20 (4) 425-445

PCR REVERSE LINE BLOT



Bergmans et al. CMI 2008, 14:778-88

Dermatophyte Detection PCR Assays IV

PCR - ELISA

- Onychodiag® kit
  Άγνωστος στόχος
  Savin et al. JCM 2007

- PCR ELISA DIG Detection kit ® *Roche*
  Topoisomerase II
  Beifuss et al. Mycoses 2010

Sensitivity of dermatophyte PCR assays

Generally, PCR based assays are considerably more sensitive than conventional methods

All PCRs reported are detecting more positive samples than mic./cul.

In a recent nail study, conventional methods had a positivity rate of 22.9% and PCR a positivity rate of 41.5%.

Brillowska-Dabrowska et al, J Clin Microbiol; 45: 1200-1204.

2 - 20 genomes detected per sample in the published studies

Does the increased sensitivity lead to more false positive results?

Positive culture results are regarded *de facto* as true positive

Need for more PCR-based studies, based on repeat sampling and outcome evaluation needed

Specificity

- Smaller number of species identified by PCR than by culture, e.g. no geophilic species have been yet detected in clinical studies

- PCR methods sometimes unable to distinguish closely related species, e.g. *T. rubrum* / *T. soudanense* / *T. violaceum* or *T. tonsurans* and *T. equinum*

ISHAM Working Group

PCR-based diagnosis of Dermatophytic infections:

on the way to a consensus

Objective

To work towards forming a consensus on standard PCR-based diagnostic methodology, able to complement or even replace the current standard in dermatophyte diagnosis, direct microscopy and culture.

Thank you!

Athos, Holy Mountain