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A journey through onygenalean families *Arthrodermataceae* and *Ajellomycetaceae*

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Faculteit der Natuurwetenschappen, Wiskunde en Informatica

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CHAPTER 1

General introduction: *Onygenales*, the order of dermatophytes and dimorphic fungi

Parts of this chapter were published in

Yanping Jiang, Karolina Dukik, Jose F. Muñoz, Lynne Sigler, Ilan S, Schwartz, Nelesh P. Govender, Chris Kenyon, Peiying Feng, Bert Gerrits van den Ende, Benjamin Stielow, Alberto M. Stchigel, Hongguang Lu, Sybren de Hoog. Phylogeny, ecology and taxonomy of systemic pathogens in *Ajellomycetaceae* (*Onygenales*): *Blastomyces, Emergomyces, Emmonsia, Emmonsiellopsis*

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Studies in Mycology (2018) 89:153-175

Historical overview of the order *Onygenales*

Members of the order *Onygenales* (*Ascomycota*, *Eurotiomycetes*) are common in nature; mostly found as saprobes in soil on keratin-rich substrates, or associated with vertebrates causing dermatophytoses and other infections. Species in this order are characterized by smooth or appendiculate, brightly colored ascomata, with walls of interwoven or anastomosing hyphae. The asci are unitunicate, subglobose to turbinate, with one-celled hyaline ascospores (1). The asexual states usually have a combination of arthric and thallic conidia. In classifying *Onygenales*, ascomata structure was considered to circumscribe the taxa in the order (2-5). However, Currah (1) pointed out that resemblance of peridial structure might be a result of convergent evolution, not a feature of the order as phylogenetic lineage. He revised the order by adding physiological features to the ascospore and conidial morphology and established four families: *Arthrodermataceae*, *Gymnoascaceae*, *Myxotrichaceae* and *Onygenaceae*. Currah's system has been widely accepted among mycologists. Three out of these four families, *Onygenaceae*, *Arthrodermataceae* and *Gymnoascaceae* are considered as core families in *Onygenales,* being consistently found as part of this order in many studies. Currah himself suggested that the family *Myxotrichaceae* was dissimilar from other onygenalean families because its affinity to plant debris and its cellololytic nature (6).

Molecular techniques employed for systematics of the order *Onygenales* proved to provide a reliable tool for revealing the phylogenetic relationships among onygenalean families. In one of the first studies based on SSU rDNA locus (7) the monophyletic nature of *Onygenaceae*, *Arthrodermataceae* and *Gymnoascaceae* was confirmed. Many subsequent molecular studies based mostly on SSU, LSU or ITS loci confirmed the monophyletic nature of these three families (6, 8-12).

Along with *Myxotrichaceae*, other families considered as onygenalean were *Eremascaceae* (8, 13), *Amauroascaceae* (6, 8, 13, 14), *Arachnomycetaceae* (8), *Ascosphaeraceae* (9, 15-17), *Nannizziopsiaceae* (10) and *Spiromastigaceae* (13). The phylogenetic relationships of some of these families with respect to the core onygenalean families remain to be answered.

In many phylogenetic studies, another group of pathogenic fungi was consistently forming its own clade within *Onygenales*. Almost 25 years ago, Leclerc et al. (18) suggested that pathogenic *Onygenales* should be separated in two groups, dermatophytes and geophilic fungi in one and systemic human pathogens in another group. These systemic fungi were clustering in one out of three subgroups in the family *Onygenaceace* (6). Other studies supported *Ajellomyces* as separated group (9, 19-21). They were established as the family *Ajellomycetaceae* by Untereiner (22). This family can be considered as fourth core family of this order.

Arthrodermataceae

Arthrodermataceae is a family of dermatophytes, the etiological agents of dermatophytoses. These infections, also known as tinea or ringworm, are generally cutaneous, invading the skin, nails and hair of humans and animals. Traditionally, the infections are described according to the infected body part as tinea capitis (infection of the scalp), tinea corporis (infection of glabrous skin), tinea cruris (groin infection), tinea pedis (foot infection), tinea manuum (hands) and tinea unguium (nails).

Dermatophytes are found worldwide; they are among the most frequently observed organisms in biomedicine. Almost every human will be infected by a dermatophyte at some point over the course of his/her life (23), resulting in more than $ 500.000 000 USD total costs per year (23, 24). They are spread by contact, from infected human to human (anthropophilic), from animals to humans (zoophilic) or from soil to animals or humans (geophilic). The infections

caused by anthropophilic species are mild, chronic and non-inflammatory with a potential to cause epidemics, while zoo- and geophilic species cause acute and inflammatory infections. Humans infected by zoophiles remain contagious which lead to self-limiting outbreaks (25) while the infections from geophilic species are singular and quickly resolved. Geo-, zoo- and anthropophilic species are considered as broad ecological groups with respect to their habitat, even though for some species there is no clear cut. This is often a case for geophilic species with reservoir in the soil around burrows of terrestrial animals which can be carried in their fur; hence they can be considered as both geo- and zoophilic. The presence of sexual stages is another parameter defining these groups, with anthropophiles being completely asexual and geophiles showing vigorous mating. The type of infection caused by these different groups has a clinical significance in terms of locating the source of infection, possible route of transmission/ spreading and deciding upon the therapeutic regimen.

The widely accepted morphology-based taxonomy of dermatophytes in the genera *Trichophyton*, *Microsporum* and *Epidermophyton* established by Emmons (26) corresponds only partly to this ecological grouping; anthropophiles and some zoophiles belong to *Trichophyton* and *Epidermophyton*, *Microsporum* is considered mostly zoophilic while geophytes belong to the genus *Arthroderma*. The species of the genus *Trichophyton* are the most prevalent and hence the most studied group of fungi due to their pathogenicity to humans (27). Of these, *Trichophyton rubrum* constitutes the majority of isolates causing dermatophytoses followed by *T. tonsurans* and *T. mentagrophytes* (28). Phylogenetic studies based on the ITS locus (23) have shown that the genus *Trichophyton* is polyphyletic. Re-classification of the *Arthrodermataceae* resulted in establishing seven genera, of which only species of *Trichophyton* and *Epidermophyton* are considered as truly anthropophilic. This phylogenetic / ecological rearrangement led to a distinction between common clinically important dermatophytes and members of other genera occasionally infecting humans.

Ajellomycetaceae

The members of *Ajellomycetaceae* are pathogenic, vertebrate-associated fungi that cause infections upon inhalation and endogenous reactivation. They are considered as environmental pathogens, i.e. having an environmental life cycle in addition to a host-associated cycle. Their infections are systemic; their point of entry is usually the lungs, and then spread via the bloodstream to multiple organs including the skin, often causing multiple organs to fail and eventually resulting in the death of the patient. These fungi affect people with normal immune function as well as those who are immunocompromised, although infections in otherwise healthy patients have a mild, self-limiting course. During the last decades systemic mycoses emerge as a global problem due to the growing population of immunocompromised patients. They may be fatal in susceptible patient populations unless diagnosed early, patients being administrated with specific antifungal therapy.

Most members of *Ajellomycetaceae* reside as filamentous forms in soil or guano and undergo morphological shift upon the inhalation by the host. This thermal dimorphism is exhibited as yeast growth *in vivo* and *in vitro* at 35-37 °C (29) and it was experimentally proven by culturing these species at 25 °C and 37 °C (22, 29-33). Nearly all species described thus far share morphological and ecological features of this dimorphism. This family comprises the species of the teleomorphic genus *Ajellomyces*, encompassing the anamorphic genera *Blastomyces*, *Emmonsia* and *Histoplasma* (22). Phylogenetic studies based on rDNA SSU, added the genera *Paracoccidioides* and *Lacazia* to the family (6, 34).

The major differences between the genera are in their pathogenic forms and to a lesser extent in their host preferences and clinical symptoms. *Blastomyces*, already known since 19th century

(35) is mostly isolated from rich soils in Canada and USA (36, 37). The fungus mostly causes pulmonary infection. Its invasive form consists of broad-based budding cells. Known species are *B. dermatitidis* and *B. gilchristii*. *Histoplasma capsulatum* was discovered in 1906 (38). The fungus was isolated from different but usually sheltered environments: caves, attics, pigeon coops. Histoplasmosis is manifested as chronic pulmonary disease or disseminated infection (39). The invasive phase consists of small yeast cells multiplying inside the macrophages. *Histoplasma* has worldwide distribution; known species (or varieties) are *H. capsulatum,* and the African species *H. duboisii*, and *H. farciminosum* infecting donkeys. *Paracoccidioides* was discovered almost at the same time as *Histoplasma* (40). This fungus is restricted to subtropical Latin America (41). Its invasive form consists of cells with multilateral budding. A possibly natural host of this fungus is the primitive terrestrial armadillo found in Latin America. There are two described species: *P. brasiliensis* and *P. lutzii*. Lacaziosis, a mycosis caused by *Lacazia loboi* is an exceptional fungus because it is acquired through cutaneous inoculation. It is observed in humans and dolphins (34) and it cannot be cultured, therefore it is considered as an obligate pathogen. The species of *Emmonsia* have cosmopolitan distribution and cause lung infections mostly in small rodents and occasionally humans (31, 42). They cause adiaspiromycosis, the invasive form consisting of large, thick-walled adiaspores. *Emmonsia crescens* and *E. parva* are two species differing in the size of the adiaspores. Many phylogenetic studies performed with these genera revealed that *Blastomyces*, *Histoplasma*, *Emmonsia* and *Paracoccidioides* belong to one clade with *Emmonsia crescens*, and *Emmonsia. parva* being close to *Blastomyces* (31, 43, 44).

Barcoding and phylogenetic markers

Rapid and accurate identification of clinically important fungi is crucial in order to set up a therapy. Many studies have been conducted in an effort to find rapid, accurate and cost-effective methods for recognition of dermatophytes and systemic dimorphic fungi. The classical methods are based on clinical symptoms and phenotypic characteristics such as culture characteristics, macro- and microscopic features and physiological tests. In the majority of hospitals, classical methods of identification based on fungal morphology are still in use. These methods are time-consuming and laborious and require highly trained staff (45). Besides, in the last decades of the 20th century it became obvious that using morphological features only has limitations in taxonomic resolution and could not be used for classification or for identification.

Since the 1980s, new molecular identification techniques have been developed which challenged the old phenotypic methods. Since their introduction, molecular identification approaches have become the methods of preference, being more accurate, easier and cheaper (46). Nowadays, molecular identification means use of standardized DNA markers, a concept known as DNA barcoding (47-50). In 1990, White et al. (51) published a key study on amplification and sequencing of the ITS spacer of the rDNA operon, describing primers for simple and robust PCR amplification and Sanger sequencing of the partial operon. Broad acceptance was achieved by generating thousands of sequence entries in public repositories leading to ratification of ITS as official fungal barcode by the fungal barcode consortium (50, 52). As a result, an overwhelming number of phylogenetic studies were performed in fungal taxonomy, including clinically important fungi, and high numbers of papers on ITS identification, classification and phylogeny of dermatophytes (53-57) and dimorphic fungi (44, 58-60) were published. However, despite the obvious strength and robustness of ITS as universal barcode for fungi, some studies have demonstrated that ITS is not always able to resolve higher taxonomic ranks, or solve clade-specific questions by generating well-supported phylograms. Most mycologists agreed that ITS locus should be combined with secondary or tertiary specific barcodes. Thus the search

for more specific barcodes and phylogenetic markers continued, mostly among protein coding genes, which exhibited better resolution than ITS (46, 61, 62).

In dermatophytes, the majority of work was based on the sequencing of ribosomal DNA genes: small subunit region (63), large subunit region (18) and the internal transcribed spacer regions or ITS (53-56, 64). Protein coding genes were tested as well, including chitin synthase I (65), β-tubulin and translation elongation factor I-α (66) or their combinations (67).

Studies on dimorphic fungi were performed in a similar manner with one or combinations of ITS, LSU and SSU regions. Guého et al. (43) used partial LSU for molecular taxonomy of *Blastomyces* and *Histoplasma*, Peterson and Sigler (1998) used ITS to elucidate the phylogenetic relationship of *Emmonsia*, *Blastomyces* and *Histoplasma*, Motoyama et al. (68) used ITS and LSU to identify *Paracoccidioides brasiliensis*. Untereiner et al. (2002) used partial LSU and mitochondrial SSU for the phylogeny of *Ajellomyces* and related taxa in *Onygenales* (9) and in 2004 established the family *Ajellomycetaceae* using ITS and LSU (69). The molecular phylogenetic study to determine the phylogenetic position of *Lacazia loboi* was based on ITS sequences (70).

In 2015, a large study including more than 1500 fungal species was conducted, testing novel candidate primers corresponding to the fungal-specific translation elongation factor 3 (*TEF3*), a small ribosomal protein necessary for t-RNA docking, the ribosomal protein *60S L10* (*L1*), DNA topoisomerase I (*TOPI*), phosphoglycerate kinase (*PGK*), hypothetical protein *LNS2* and alternative sections of *TEF1*α as potential universal or secondary barcodes to the ITS barcoding locus (71). Assessing their identification and phylogenetic power alone or in combination with the rDNA operon and standard protein-coding loci should answer unresolved phylogenetic relationships among the taxa, and reveal phylogenetic positions of known species, and discover cryptic species.

Locus assessment for the family *Arthrodermataceae*

Prior to conducting multilocus phylogeny of dermatophytes, a subset of 123 strains was tested with nine sets of primers for eight DNA loci. The nine alignments obtained from the initial dataset of 123 strains using MAFFT v. 6.850b (72) were subjected to phylogenetic analysis using maximum likelihood (ML) in Mega v6.0 software. For an assessment of primer sets and resulting phylogenetic trees, we employed four parameters to select loci with superior identification and phylogenetic power and to exclude the ones with poor performance: (1) PCR robustness, (2) number of obtained amplicons, (3) total number of supported clades (BS>80 %) in trees, and (4) monophyly of the genera. Based on these parameters, *TUB2, RP 60S L1,* and *TEF*3 were chosen for further analysis along with the standard ITS and LSU loci.

In the second analysis, 141 strains were added (total data set of 264 strains), for which the five chosen loci were analyzed. From the total set of 264 strains, five alignments of successfully amplified sequences were created, containing *n*=238 (ITS), *n*=219 (LSU), *n*=198 (*TUB2*), *n*=211 (*TEF3*) and *n*=222 (*RP 60S L1*) sequences, respectively (Table 1). The alignments were analyzed using Raxml v8.0.0 and MrBayes v.3.2.6 analysis (Cipres portal, https://www.phylo.org/) with additional Raxml v8.0.0 analysis (R147) on the five alignments containing 147 sequences from successfully amplified loci. (Table 2). The highest number

Table 1. Robustness of phylogenetic trees for ITS, LSU, *TUB2, TEF3,* and *RP 60S L1.*

Locus	ITS	LSU	*TUB2*	*TEF3*	*RP 60S L1*
n = amplicons*	238	219	198	211	222
n = clades†	40	17	29	17	30

*Number of amplicons per locus for the data set of 264 strains; † number of clades with bootstrap value BS>80 % in phylogenetic trees obtained from 147 strains possessing all 5 amplicons.

Table 2. Assessment of phylogenetic trees for ITS, LSU, *TUB2, TEF3*, and *RP 60S L1* obtained by Maximum Likelihood in RAxML v8.0.0 and MrBayes v.3.2.6. Numbers represent bootstrap supports and posterior probabilities of the clades higher than 80% and 0.9 (BS>80%, PP>0.9) respectively, representing the genera.

Locus	**ITS**			**LSU**			***TUB2***			***TEF3***			***RP 60S L1***		
software	**B238**	**R147**	**R238**	**B219**	**R147**	**R219**	**B198**	**R147**	**R198**	**B211**	**R147**	**R211**	**B222**	**R147**	**R222**
T	1	100	100	X	X	X	1	99	100	0	0	0	1	98	100
E	1	100	100	1	100	100	1	100	100	1	100	100	1	100	94
N	0.98	85	84	X	X	X	X	X	X	X	X	X	X	X	X
P	1	100	100	0,99	X	86	0,99	0	86	X	X	99	1	100	X
M	1	100	100	1	99	99	1	100	99	1	100	100	1	100	100
A	1	99	95	0	X	83	0,99	95	0	X	X	X	X	0	0
L	1	ND	100	0,97	ND	0	ND	ND	ND	ND	ND	ND	1	ND	100

Abbreviations used: R = RAxML v8.0.0 software; B=MrBayes v.3.2.6 software; T = *Trichophyton*; E = *Epidermophyton*; N = *Nannizzia*; P = *Paraphyton*; M = *Microsporum*; A = *Arthroderma*; L = *Lophophyton*; ND = no data; X = no clade.

of supported clades (*n*=40) was found in the ITS tree, followed by *RP 60S L1* (*n*=30)*, TUB2* (*n*=29), and LSU (*n*=17) and *TEF3* (*n*=17) (Table 1). Monophyly of seven ITS-defined genera (*Arthroderma, Epidermophyton, Lophophyton, Microsporum, Nannizzia, Paraphyton,* and *Trichophyton*) in *Arthrodermataceae* was taken as lead for attributing clades for the other genes using the three analyses mentioned above (Fig. 1). ITS trees were stable, independent from size of data sets or used algorithm. Genus *Nannizzia* was a supported clade in all three ITS analyses, though with lower bootstrap and posterior probability values than the other genera. In none of the remaining trees was the genus *Nannizzia* recognized as a monophyletic group. Poor resolution was achieved with *TUB2* and *RP 60S L1,* while LSU and *TEF3* had very poor performance as phylogenetic markers. Since ITS locus produced by far the largest number of supported clades, taking another gene as reference would not yield trustworthy results. Therefore, datasets of ITS sequences were concluded to be superior compared to the other four loci, decomposing the entire tree in clades with BS > 80 %. The tree of concatenated sequences of ITS, *TUB2, RP 60S L1* and LSU (**chapter 2**) had similar topology as ITS tree alone.

Locus assessment for the family *Ajellomycetaceae*

Multilocus phylogenetic studies of *Ajellomycetaceae* were performed using ITS, LSU, *TUB2*, *TEF3* and *RP 60S L1* (**chapter 5**) and alternatively using locus *rPB2* instead of *RP 60S L1*. The information density of ITS, LSU, *TUB2*, *TEF3* and *rPB2* loci individually was assessed in order to infer: (a) which marker shows the best potential to recognize the described taxa, thus maximizing affiliation to an existing taxon e.g. in the case of an unknown clinical sample, and (b) which sections of the here studied genes are most informative and render taxa with the highest probability as monophyletic. The R packages SPIDER and APE were used to assess these density metrics over all loci. Fig. 2 outlines subsequently the following plots for each locus: (A) 'congruence of NJ (neighbor joining) trees', (B) 'proportion of species that are monophyletic' and (C) 'sum of diagnostic nucleotides'. The first metric describes the proportion/quantity of neighbor joining trees being congruent (identical) to each other for a given sliding window (here defined with 100 nucleotides), and thus is a consistency metric for statistical resampling. It describes the robustness of the given section for a given locus to retrieve the same species affiliations. The second metric describes in a similar way to the first metric, how consistent taxa could be rendered as monophyletic entities over the statistical resampling process. The third

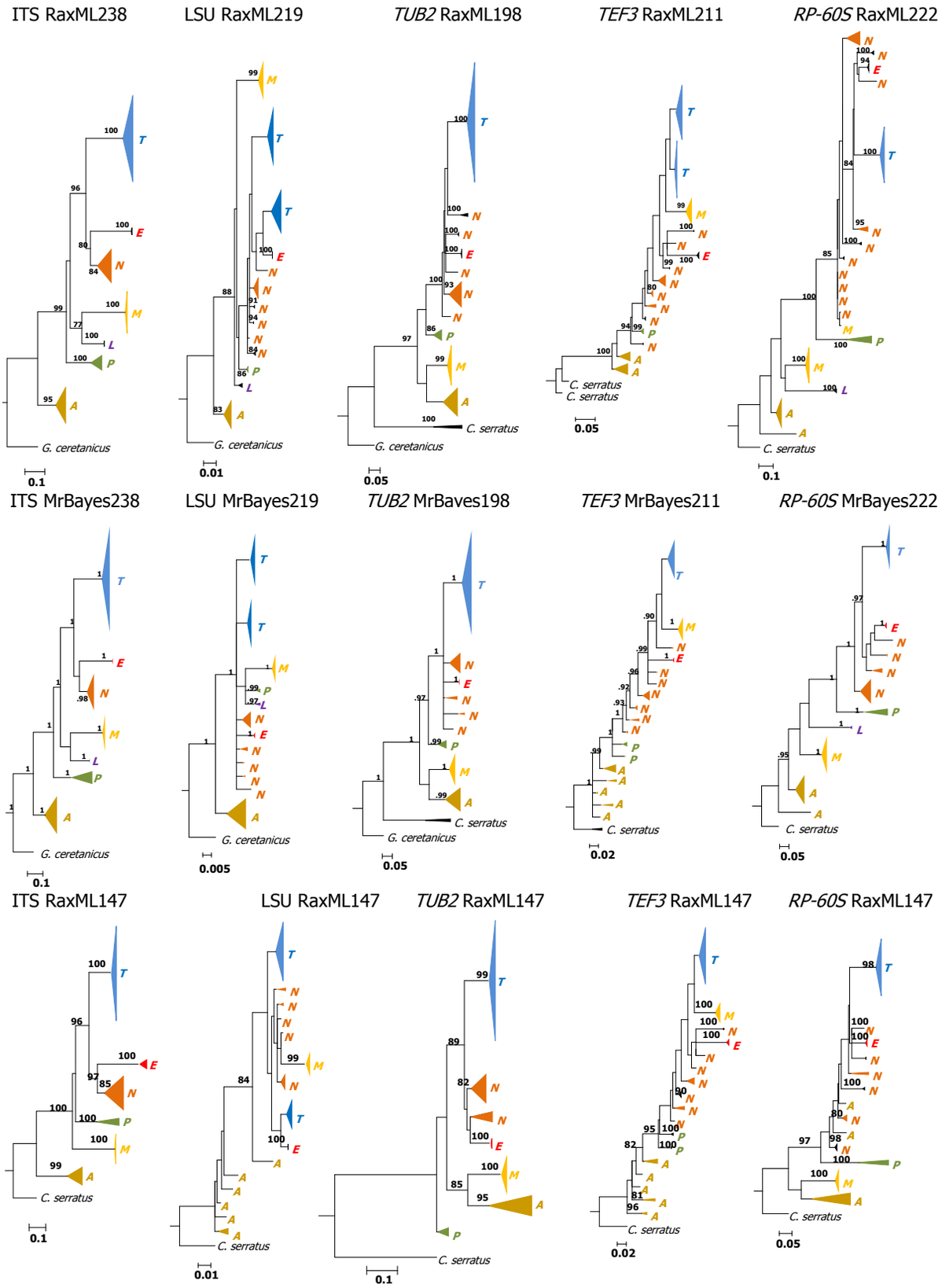

Figure 1. Comparison of five gene-trees based on maximum datasets of strains analyzed (ITS n=238, LSU n=219, *TUB2* n=198, *TEF3* n=211, *RP-60S L1* n=222), compared with a set of strains for which all genes were sequenced (n=147). Phylogenetic analysis was done with RAXML, MRBAYES, using *Guarromyces ceretanicus* or *Ctenomyces serratus* as outgroup. Bootstrap values > 80 % are shown with the branches.

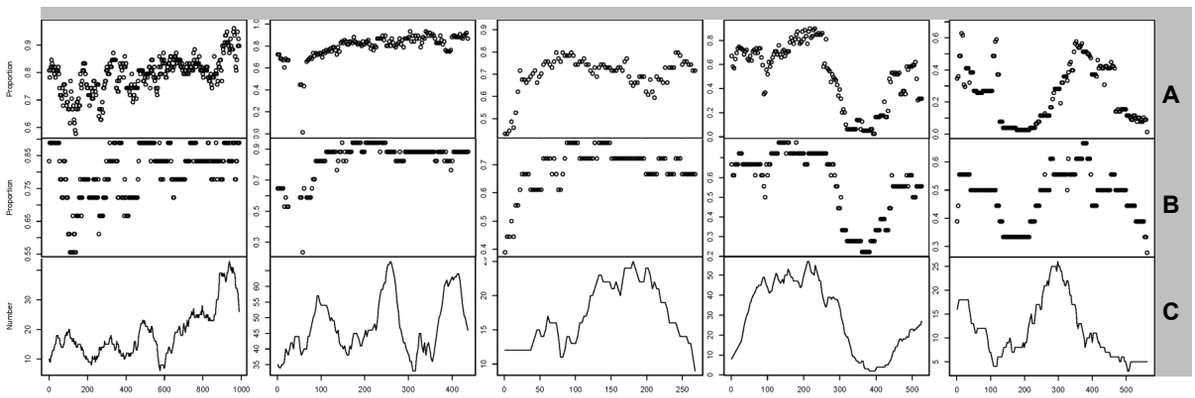

Figure 2. Sliding window analysis conducted with the R package SPIDER, covering a 100 bp sliding-window for the analyzed partial gene sequences (multiple alignment) respectively. A. Congruency of neighbor-joining (NJ) trees, when re-sampled 1000 times over each window. B. Proportion of species that are monophyletic, indicating quantity of discrete species entities derived from NJ re-sampling process and as defined in this study. C. Sum of diagnostic nucleotides at each window position corresponding to the degree of informativeness given a particular alignment position. Note: scaling of Y-axis is not proportional, values range from 0 (lowest) to 1 (highest) for A and B respectively, for C, number indicates quantities of diagnostic nucleotides for a given window.

metric is quantitatively describing how many nucleotides per window position are informative/diagnostic, thus describing the information content numerically. Values for the first and second metric range from 0 to 1, with 0 no NJ trees/no species being congruent/monophyletic respectively, while 1 is to be interpreted as the opposite, and for the third metric range is from 0 to infinitive. Fig. 3 shows inter-specific (orange whiskers) K2P distances over the intra-specific K2P distances (blue whiskers) over each window. Ideally, orange and blue whiskers are entirely separated. Taxa are sufficiently well resolved when intra-specific distances never exceed closest non-conspecific (inter-specific) pairwise distances. In other words, this is the 'classical' barcoding gap (48).

The informativeness of the analyzed loci was relatively even between the individual loci, thus none of the genes exposed a higher inter-specific distance over a lower intra-specific distance between the analyzed taxa. However, clear qualitative differences were observable with particular attention to (a) the informativeness of the individual sequence section analysed, and (b) information density over a particular section analysed. When referring to the informativeness of the individual sections (NJ tree congruency/Proportion of monophyletic species), the ranking is the following: *TUB2* > *rPB2* > ITS/*TEF3* (approximately equal) > LSU. *TUB2* here provides the highest consistency over the entire sequence analyzed, as revealed in Fig. 3 and the corresponding

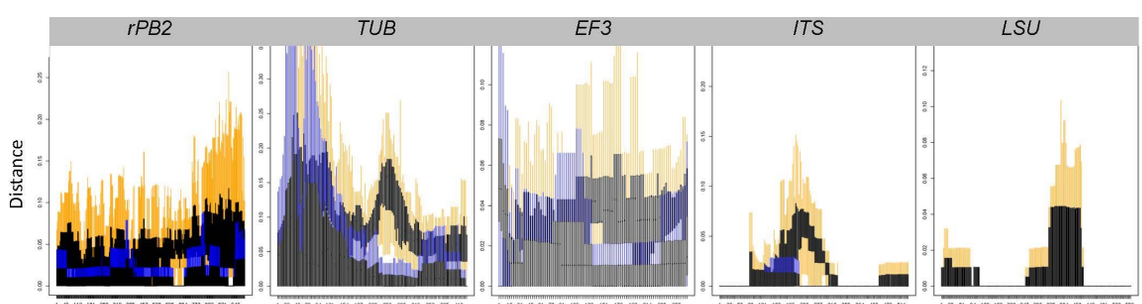

Figure 3. 'The barcoding-gap' represented as a 100 bp sliding-window for each analyzed partial gene sequences (multiple alignment) respectively. Boxplots for each nucleotide position are shown, indicating closest non-conspecific distances (orange whiskers) and intra-specific distances (blue whiskers). Y-axis indicates pairwise distance (K80), and X-axis window (alignment) position. Ideally, orange and blue whiskers are entirely separated. Taxa are sufficiently well resolved when intra-specific distances never exceed closest non-conspecific (inter-specific) pairwise distances.

plots for 'NJ tree congruency/Proportion of monophyletic species', particularly since the latter values are consistently > 0.8 from ~90–450 bp of the sequence matrix. A similar result was obtained for *rPB2*, which scores high in its information content. Complete *rPB2* was determined as slightly superior over any of the other four partial gene sections for recognition of the here studied fungi. In contrast to these positive results, surprisingly only the ITS-1 of the gold standard DNA barcode renders NJ-trees/taxa proportionally consistent, while the ITS-2 section performs poorly and indicates almost a complete drop-out as is revealed in Fig. 3, and in the corresponding plot 'Sum of diagnostic nucleotides' (section ~250–550 bp). While the fungal specific *TEF3* also exposes a relatively high consistency, it overall ranks lower (or similarly equal to ITS) due to a lower ratio of diagnostic nucleotides, e.g. when compared to *TUB2*. The most uninformative locus, at least at the species level, was partial LSU; all sliding window plots revealed that only a small section, approximately between 250 and 350 bp contains a sufficiently variable number of nucleotides to recognize the taxa studied here. However, maximum consistency between NJ trees/monophyletic taxa is approximately half of the best two loci *TUB2/rPB2*.

Phenotypic (classical) methods of identification

For fungi that are causative agents of human and animal infections, their rapid and accurate identification in clinical laboratories is of utmost importance. Nowadays, molecular methods, referring mostly to ITS sequencing are considered as a gold standard for identification of clinically relevant fungi. However, molecular approaches may fail to identify fungi which are genetically indistinguishable but have profound differences in morphology and clinical symptoms. Examples of well-established clinically different species are known among dermatophytes. For such species, combining molecular with phenotypic characters is needed for accurate identification.

Phenotypic characters include culture and microscopic morphology such as growth rate, colony texture and pigmentation, size and shape of macro- and microconidia as well as several physiological properties. The morphotaxonomy of dermatophytes established by Emmons (26), based on study of vegetative structures and conidia, synonymized some species that were lately recognized as distinct entities when their physiological and/or molecular features were established.

In clinical settings, the dermatophyte nature of the etiological agent of infection can be confirmed by growth of the fungus on Dermatophyte Test Medium (DTM) (73). Weitzman et al. (74) introduced trichophyton-agars, utilizing strain ability to assimilate a panel of essential vitamins. Other commonly used physiological tests for dermatophytes are *in vitro* hair perforation test, production of urease on Christensen's urea broth, tolerance to NaCl and cycloheximide, hydrolysis of milk solids on BCP-MS-G (75), hemolysis on blood agar (BioMérieux), and lipase activity on TOTM agar (76). The physiological features of dermatophytes are meant to be used as addition to their morphology, not as a sole method of identification.

Alternative identification methods: Mass spectrometry

Another group of molecular methods based on the analysis of proteins has been developed for identification purposes. Mass spectrometry is based on analysis of proteins instead of nucleic acids. The identification of microorganisms entered a new era with the introduction of the MALDI-TOF mass spectrometry analysis as an alternative to DNA-dependent methods (77). In short, MALDI-TOF generates spectra that can be viewed as a protein fingerprint signature of a given microorganism. When there is a match between the mass spectra of a microorganism and the mass spectra in a reference database, the microorganism is considered to be identified (78).

This new technique was introduced first as a successful identification method for bacteria (79, 80) and for yeasts (81-83). With the successful implementation of MALDI-TOF in bacterial and yeasts identification a question was opened if this method can be used for identification of clinically important fungi. In the last decade, many research groups were developing and testing MALDI-TOF on different groups of clinically important fungi (84-88). Some of these studies were focused on dermatophytes (89-95) and to lesser extent on dimorphic fungi (96, 97).

Another method that can be used for identification purposes is LC-MS/MS, liquid chromatography tandem mass spectrometry. This is a technique in which the separation power of the liquid chromatography is coupled with the analytical power of mass spectrometry. With LC the proteins or peptides from complex mixtures are separated and as such they are electro-sprayed for mass spectrometric analysis. MS/MS stands for tandem mass spectrometry where there are two levels of analysis. The first level (MS1) is based on the molecular weight of the proteins or peptides, whilst MS2 is analyzing the fragmentation pattern of the chosen proteins. In this way, several thousands of highly informative MS/MS spectra from the fragmented peptides or proteins are obtained. The main difference between LC-MS/MS and MALDI-TOF is that the latter is based on protein fingerprinting, while LC-MS/MS is based on sequencing individual peptides. The outcome from MALDI-TOF fingerprinting is a list of candidates with a ranking score, while with LC-MS/MS 100 identification of a protein is achieved with 100% reliability. Theoretically, LC-MS/MS can identify thousands of proteins in a mixture with the same reliability as for a single protein. Every single one of these identified and characterized proteins can be used for distinction purposes, for example as identification markers.

Within LC-MS/MS, there are two fundamental strategies for protein identification and characterization: bottom-up and top-down approaches. In the bottom-up approach purified proteins or complex protein mixtures are subjected to proteolytic cleavage, and the peptide products are analyzed by MS. In top-down proteomics, intact protein ions or large protein fragments are subjected directly to tandem mass spectrometry. The main advantages of the top-down analysis are the ability to detect sequence variants and the (combinations of) post translational modifications.

The LC-MS/MS technique has been widely used in the last decades of the 21th century in clinical laboratories for drug discovery, toxicology, endocrine laboratories etc. In the fungal world, this technique has found its place in analysis of filamentous fungi used in biotechnology (98). Shotgun proteomics, which is a bottom up approach, has been used to describe the conidial proteome of *Trichophyton rubrum* (99). Giddey et al. (100) used a bottom-up approach to analyze secreted proteins from both *T. tonsurans* and *T. rubrum* complexes. These approaches have not been used for identification purposes.

In vitro antifungal susceptibility

Antifungal susceptibility testing is an essential tool for clinicians to guide the antifungal therapy. Three main families of antifungals are used in clinical settings to treat fungal infections. First, polyenes are represented by amphotericin B and its different formulations which exert their activity via binding to ergosterol in the fungal cell membrane. Furthermore, azoles comprise several derivatives such as itraconazole, fluconazole, voriconazole, posaconazole, isavuconazole. They block the demethylation of lanosterol therefore inhibiting ergosterol synthesis. Finaly, caspofungin, micafungin and anidulafungin are echinocandins that inhibit the synthesis of 1,3-beta-glucan in the cell wall.

In the last 15 years, a lot of work has been done in this area of research, in an effort to standardize the methods for testing. Standardization of *in vitro* susceptibility tests by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial

Susceptibility Testing (EUCAST) are major steps in this field (101). The Clinical Laboratory and Standards Institute (CLSI) has released methods for testing of both yeasts and filamentous fungi (102, 103)
MIC breakpoints have been established for *Candida* spp. and *Aspergillus* species, the most frequent agents of fungal infections in the world. *Cryptococcus* and *Pneumocystis* together with *Candida* and *Aspergillus* are also among the main etiologic agents of fungal infections (104), mainly in immunocompromised individuals. On the other hand, dimorphic fungi such as *Histoplasma, Blastomyces, Coccidioides* and *Paracoccidioides* can affect immunocompetent patients as well, causing endemic mycoses which range from subclinical respiratory infections to life-threatening systemic disease. In general, reference methods for antimicrobial susceptibility tests follow those established by the CLSI. Notably missing from these reference methods are procedures specifically designated for dimorphic fungi, leading to confusion as to whether macro- and microdilution test methods for yeasts (M27-A3) (ref) or for filamentous fungi (M38-A2) should be followed. This is not an insignificant question, as the yeast and hyphal forms of the dimorphic fungi can have dramatically different susceptibilities (105). Some studies of different endemic fungi using mycelial and yeast forms for susceptibility testing resulted in opposite conclusions (106, 107). In the last two decades, new emerging dimorphic fungi have been causing disseminated infections mostly in HIV-infected patients. There were many reports of such infections mostly in South Africa (108) and in some other parts of the world (109-113). Antifungal susceptibility testing of these newly emerging fungi is of prime importance in guiding the therapy for these patients.

Outline of the thesis

The two families of onygenalean pathogenic fungi, *Arthrodermataceae* and *Ajellomycetaceae,* are the focus of this thesis. Their monophyly within *Onygenales* has been confirmed in many studies till now, but the phylogenetic relationship between the taxa within the families and delineation of the species was not completely resolved. During the last decades of the molecular era, many loci have been tested for their phylogenetic and identification power. What came up as general assumption is that phylogenetic analyses based on a single locus should be taken with caution, especially for taxa delineation and taxonomic conclusions. The main goal of this thesis was to establish a stable taxonomy for both *Arthrodermataceae* and *Ajellomycetaceae* through multilocus phylogenetic analyses based on representative sampling. Five loci were chosen for phylogenetic studies based on a previous locus assessment presented in **chapter 1**. **Chapters 2** and **5** represent multilocus phylogenies of *Arthrodermataceae* and *Ajellomycetaceae* respectively. With both phylogenetic studies the taxonomy of these two families has reached an acceptable level of stability. In **chapters 3** and **4**, LC-MS/MS mass spectrometry and a polyphasic approach, respectively, were utilized to compare/combine them with ITS and multilocus sequencing and to establish species boundaries with certainty. *Trichophyton rubrum* / *T. violaceum* and *T. tonsurans* / *T. equinum* were analyzed with LC-MS/MS methodology and the species of the genus *Nannizzia* were fully characterized using polyphasic approach. Antifungal susceptibility testing of dimorphic fungi in *Ajellomycetaceae* (**chapter 6**) was performed for the first time on the newly described taxa in order to serve as guidance for clinical treatment. As a continuation of this thesis, future work might include a multilocus analysis on representative members of the whole order *Onygenales*, in order to reveal the number and the phylogenetic relationships between onygenalean families.

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CHAPTER 2

Toward a novel multilocus phylogenetic taxonomy for the dermatophytes

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Abstract

Type and reference strains of members of the onygenalean family *Arthrodermataceae* have been sequenced for rDNA ITS and partial LSU, the ribosomal 60S protein, and fragments of β-tubulin and translation elongation factor 3. The resulting phylogenetic trees showed a large degree of correspondence, and topologies matched those of earlier published phylogenies demonstrating that the phylogenetic representation of dermatophytes and dermatophyte-like fungi has reached an acceptable level of stability. All trees showed *Trichophyton* to be polyphyletic. In the present paper, *Trichophyton* is restricted to mainly the derived clade, resulting in classification of nearly all anthropophilic dermatophytes in *Trichophyton* and *Epidermophyton*, along with some zoophilic species that regularly infect humans. *Microsporum* is restricted to some species around *M. canis*, while the geophilic species and zoophilic species that are more remote from the human sphere are divided over *Arthroderma*, *Lophophyton* and *Nannizzia*. A new genus *Guarromyces* is proposed for *Keratinomyces ceretanicus*. Thirteen new combinations are proposed; in an overview of all described species it is noted that the largest number of novelties was introduced during the decades 1920–1940, when morphological characters were used in addition to clinical features. Species are neo- or epi- typified where necessary, which was the case in *Arthroderma curreyi*, *Epidermophyton floccosum*, *Lophophyton gallinae*, *Trichophyton equinum*, *T. mentagrophytes*, *T. quinckeanum*, *T. schoenleinii*, *T. soudanense*, and *T. verrucosum*. In the newly proposed taxonomy, *Trichophyton* contains 16 species, *Epidermophyton* one species, *Nannizzia* 9 species, *Microsporum* 3 species, *Lophophyton* 1 species, *Arthroderma* 21 species and *Ctenomyces* 1 species, but more detailed studies remain needed to establish species borderlines. Each species now has a single valid name. Two new genera are introduced: *Guarromyces* and *Paraphyton*. The number of genera has increased, but species that are relevant to routine diagnostics now belong to smaller groups, which enhances their identification.

Keywords

Arthrodermataceae, Dermatophytes, Phylogeny, Taxonomy, *Trichophyton*

Introduction

The dermatophytes belong to the oldest groups of microorganisms that have been recognized as agents of human disease. The taxonomy of these fungi was initiated in 1841 with the studies of Robert Remak and David Gruby (1). Between 1840 and 1875, five of the main species known today, viz. *Microsporum audouinii*, *Epidermophyton floccosum*, *Trichophyton schoenleinii*, *T. tonsurans* and *T. mentagrophytes* had already been described; this was several decades before the discovery of Pasteur's invention of axenic culture (2). The only ubiquitous modern dermatophyte missing from the list is *Trichophyton rubrum* (3), which has been hypothesized to have emerged in the twentieth century (4).

After Pasteur's time, culturing of dermatophytes and description of new species has taken off enormously. Species were defined on the basis of combined clinical pictures and morphological characters in vitro. Sixteen human-associated species were introduced between 1870 and 1920, with Sabouraud's (5) magistral overview of the dermatophytes setting a new standard. During the decades that followed, application of the new methodological standard led to an explosion of new species and recombined names (Fig. 1). Generic concepts remained confused, leading to repeated name changes with a total of 350 names around the year 1950. Subsequently anamorph nomenclature stabilized by the wide acceptance of *Epidermophyton*, *Microsporum* and *Trichophyton* as the genera covering all dermatophytes.

Culture and microscopic morphology worked well as diagnostic parameters when fresh isolates were used, but were difficult to maintain and reproduce because of rapid degeneration. Standardization with reference strains was therefore difficult, and this led to the introduction of numerous taxa that are now regarded as synonyms of earlier described species. In addition, diverse types of morphological mutants were described as separate taxa, such as *Keratinomyces longifusus*, which turned out to be *Microsporum fulvum* with strongly coherent conidia (6). This misclassification is an unavoidable consequence of a diagnostic system based on the phenotype. Similar misjudgments of mutants of a single species also occurred elsewhere, sometimes unknowingly leading to the description of a separate genus for the mutant: compare, e.g., the genus pairs *Bipolaris*/*Dissitimurus*, *Scedosporium*/*Polycytella*, *Exophiala*/*Sarcinomyces*, or *Trichosporon*/*Fissuricella* (7). In addition, several dermatophytes are known which do not or poorly sporulate in culture and thus show very limited phenotypic characteristics. Classically such species were partly based on clinical symptoms, e.g., *T. concentricum* or *T. schoenleinii*, but many more, undescribed species may exist (8).

In the last decades of the twentieth century, it became obvious that morphology had its limitations and could not be used as sole characteristic for classification or identification. Given these problems, Weitzman et al. (9) introduced an additional character set in the form of physiological parameters, so-called trichophyton-agars utilizing the ability of strains to assimilate a panel of essential vitamins, but also growth temperature, gelatin liquefaction, etc. The method now indicated as the 'conventional approach' to dermatophyte taxonomy combines clinical appearance, cultural characteristics, microscopy and physiology. Serology has never really taken off.

Biological species concepts entered the picture with the modern rediscovery of dermatophyte teleomorphs by Dawson and Gentles (10) and Stockdale (11). Several geophilic and zoophilic dermatophytes, as well as related non-pathogenic species like *Trichophyton terrestre* and *T. ajelloi*, were found to produce sexual states, for which the genera *Arthroderma* and *Nannizzia* were introduced. This led to a new boom in the number of names (Fig. 1) and marked the introduction of dual nomenclature for dermatophytes. The delineation of sexual

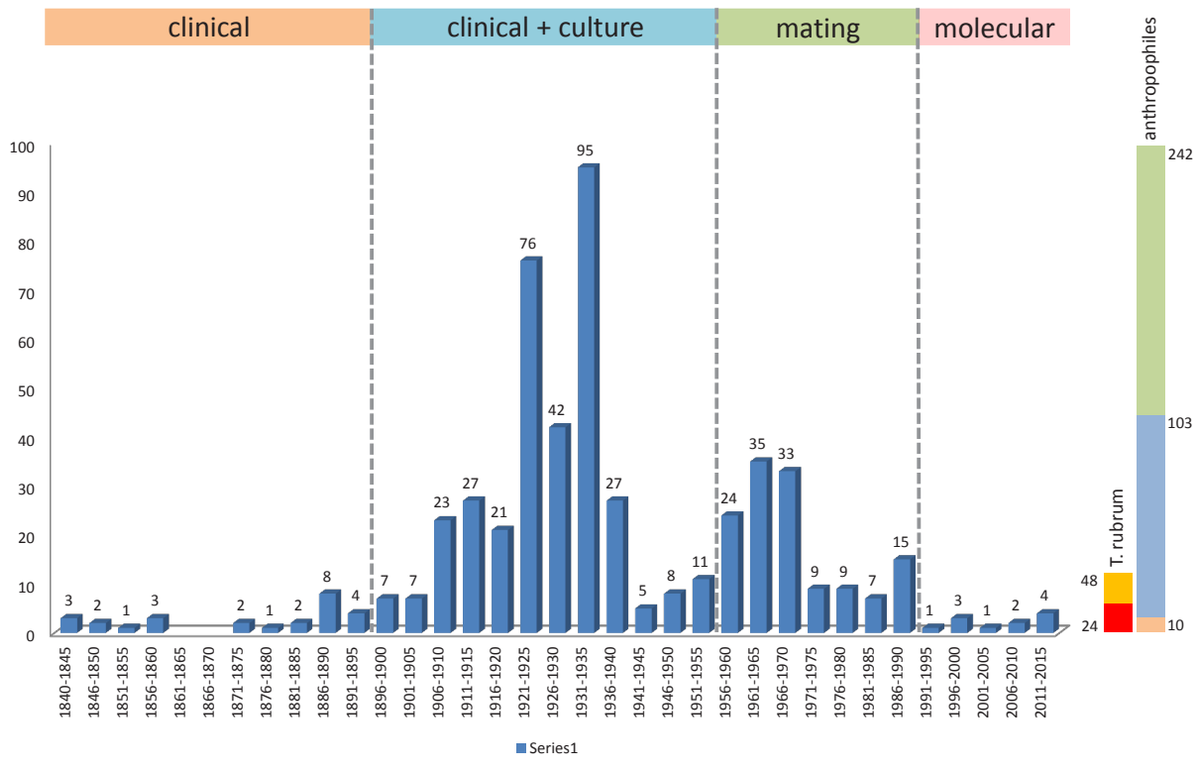

Figure. 1. Number of name changes of members of *Arthrodermataceae* during the period 1840–2015, with 5-year increments. The largest number of new names was created when morphology was added to clinical data as criteria for species distinction. The period 1960–1995 is marked by the addition of teleomorph names, leading to dual nomenclature of the dermatophytes. The bar at the right shows the approximate number of existing anthropophilic species (n = 10), the number of times these have been described (basionyms: n = 103) and the total number of name changes for these 10 species (n = 242). Possible (7) and proven synonyms of *Trichophyton rubrum* are listed in ocher (n = 48), of which (n = 24) were basionyms, in red.

interaction began to take an unusual course when Stockdale (12) discovered that members of many apparently non-mating species could be induced to reveal their mating type in an incomplete mating reaction with testers of *Arthroderma simii*. Most of the recognized asexual species could be typed in this manner and demonstrated to be descended from a single ancestral mating type. For example, *Trichophyton rubrum* was shown to be (-) in mating type, while its close relative *T. megninii*, currently considered to be synonymous, was (+). Just a few important species, such as *Epidermophyton floccosum* and *T. soudanense*, a further member of the rubrum series, resisted typing with this system and remained of unknown status. Summerbell (13) pointed out the obvious ecological factor linking the nonsexual species: they all infected animals (including *Homo sapiens*) without having a terrestrial reservoir allowing the elaborate sexual processes with ascigerous fruit bodies to take place on keratinous debris.

Clinical significance

Large differences are known to exist between species with respect to their natural habitat. Three broad ecological groups of dermatophyte species are recognized: anthropophilic, zoophilic, or geophilic (Table 1). Sometimes species cannot be clearly attributed to one of these groups due to insufficient data. Anthropophilic species naturally colonize humans, being transmitted between humans and usually cause chronic, mild, non-inflammatory infections and

often reaching epidemic proportions. Animal-carriage of these species does occur (14) but is exceptional. Zoophilic species live in close association with animals other than humans and transmission to humans usually occurs through their reservoirs. The fungi occur in the fur of particular animal hosts, either symptomatically or asymptomatically, and can become epidemic. Geophilic dermatophytes have their reservoir in the soil around burrows of specific terrestrial mammals, feeding on keratinous debris. They may be carried by these animals in their fur (15); hence, the difference between geophilic and zoophilic dermatophytes is not always sharp. When transmitted to humans, zoo-and geophilic species cause acute, inflammatory mycoses. Occasionally, humans infected by zoophiles remain contagious, leading to small, self-limiting outbreaks (16), while most infections by geophiles are quickly resolved. Thus, also in the effectivity of human-to-human transmission an increasing trend is observed from geophiles via zoophiles to anthropophiles. No sexual phases are known in truly anthropophilic species, while geophilic species show vigorous mating. By these combined parameters, the three ecological groups, although not sharply separated, are fundamentally different and also have clinical significance (Table 1).

Table 1. Broad classification of dermatophytes on the basis of ecological and clinical parameters.

	Geophilic	**Zoophilic**	**Anthropophilic**
Phylogeny	Ancestral	Moderate	Derived
Sexuality	Vigorous mating	Mostly mating	Clonal
Infection	Highly inflammatory	Moderately inflammatory	on-inflammatory
Transmission	Via environment	Double life cycle	Via host
Resolution	Quickly resolved	Resolved, self-limiting epidemics	Chronic

Experimental methods

Enabled by the recent publication of whole genome sequences of several dermatophyte species (17), idiomorphs of the mating type loci (alpha domain and HMG domain genes) were detected directly at DNA level. Using partial amplification of each locus, Kano et al. (18) were able to confirm molecularly that 22 *T. verrucosum* strains exhibited a single mating type only. Gräser et al. (unpublished data) revealed that a single mating type was present in numerous species: *T. tonsurans*, *T. equinum*, *T. interdigitale*, *T. schoenleinii*, *T. rubrum*, *T. violaceum*, *T. erinacei*, *T. concentricum*, *M. audouinii* and *M. ferrugineum*. This supports the view of clonal reproduction due to the loss of one of the mating types on species level. Some exceptions were found with zoophilic species such as *T. benhamiae* and *T. mentagrophytes*, where both types such as alpha and HMG were present with different distribution ratios (19, 20). This implies that all anthropophilic and most zoophilic dermatophytes reproduce clonally by asexual propagation in apparently stable environmental niches. In contrast, Anzawa et al. (21) showed mating of a highly competent *A. simii* tester strain producing a fertile F1 generation with a strain of *T. rubrum*, challenging the biological species concept, although only a single out of 35 ascospores proved to be a real hybrid of the two species. Apparently, the dermatophytes have held an atavistic ability to undergo genetic exchange via sexual reproduction/hybridization in response, e.g., the stressful conditions of a newly inhabited environment. In practice, due to the different ecological niches of species like the

anthropophilic species *T. rubrum* and the zoophilic species *A. simii*, they do not have the possibility to meet each other in nature.

Like in Pasteur's days, when axenic culture revolutionized microbiology, the application of molecular methods since 20 years has revolutionized dermatophyte taxonomy and that of other fungi. First molecular papers used the ribosomal small and large subunits as markers (22, 23). In a series of papers, Gräser et al. (6, 24) applied the more variable rDNA ITS region and were able to resolve a large number of species. This molecular system has been confirmed several times in later studies (25) and with different molecular markers such as ß tubuline (26, 27) and *TEF1* (28). The main topology of the *Arthrodermataceae* seems to be molecularly stable, but does not entirely correspond with morphology, as *Trichophyton* appears to be polyphyletic. As noted in earlier papers by Gräser et al. (6, 24), anthropophilic species are confined to some derived clusters, zoophilic species of domesticated mammal hosts are located in the middle of the tree, while geophilic species are located in an ancestral position, and the lower clusters are still unstable due to taxon sampling effects. For reasons of clinical understanding, it is recommendable to formalize these differences in a new taxonomic system, which is one of the aims of the present paper.

While the molecular approach was able to resolve the main traits of dermatophyte evolution, it may fail in the details. Several well-established, clinically different species such as *Trichophyton rubrum*/*T. violaceum*, *T. equinum*/*T. tonsurans* and to a certain extent also *M. audouinii*/*M. canis*/*M. ferrugineum* appeared largely indistinguishable in our multilocus analysis. Small sequencing ambiguities or missing data in this large dataset may blur the small differences very recently emerged species. Therefore, despite the available large body of research on these species, polyphasic studies combining molecular, ecological, phenotypic and life cycle data are needed to establish the validity of these species with certainty.

With the various taxonomic approaches, also nomenclatural rules have evolved over time (Fig. 2). In the nineteenth century, a clinical description was judged sufficient to characterize a fungus. Deposition of a type specimen became compulsory only in 1957. Today, the reference of a type is essential to stabilize the species' delimitation and nomenclature. Older, long-forgotten names without types are discarded as doubtful, but well-known species names should be maintained by neotypification (6). During the decades of dual nomenclature, species can have two types, but since 2013 the name, anamorph or teleomorph, always refers to the same, original type specimen. Present-day naming of fungi is according to their gross phylogenetic position. It should be realized, however, that positions in trees are relative, being dependent on the coincidentally selected constituents of the tree. Therefore, polyphasic species remain concepts essential for reliable nomenclature.

For a checklist of obsolete names in dermatophytes for which no type material is known to exist, is referred to de Hoog et al. (7). Numerous later described species were placed in synonymy, because they proved to not to deviate on the basis of modern characters. de Hoog et al. (7) listed 24 basionyms (with 48 combinations in total) as probably synonymous with *Trichophyton rubrum* (Fig. 1) (although only 5 basionyms could be proven with extant type materials). Several of the apparent synonyms were only recently segregated from *T. rubrum* on the basis of physiological parameters, which has shed doubt over usefulness of physiology as a taxonomic parameter.

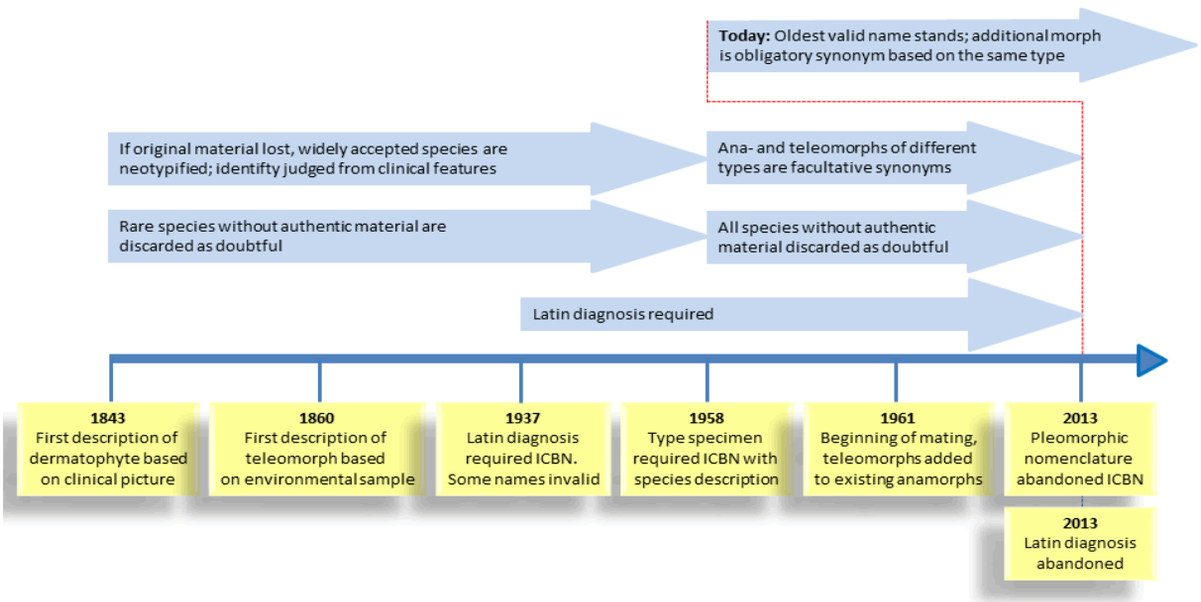

Figure. 2. Overview of changing taxonomic principles during the period 1840–2015. Of the oldest species, no original material has been preserved; the rare ones are discarded as being doubtful; the widely used names are neotypified. Latin diagnoses were required between 1937 and 2013. Pleomorphic naming with separate typification of ana- and teleomorph has been relevant between 1957 and 2013. The generic and specific nomenclatural system proposed in the present article is valid from the situation per January 1, 2013 onwards.

Materials and methods

Nomenclature

A search for possible generic names in *Arthrodermataceae* was limited to members of the order *Onygenales*. Candidate generic names were those type species in the family according to the Index Fungorum (www.indexfungorum.org). Obsolete generic names were taken from species synonyms and list of doubtful species in the Atlas of Clinical Fungi (7). For every taxon to be accepted as a potential name or synonym, permanently inactivated (dried or under liquid nitrogen) holotype material had to be necessary. Holotypes as well as living strains connected with the holotypes were indicated as type (T). In heterothallic species, mating partners needed to obtain the teleomorph were listed as syntypes (ST). Taxa without types were discarded as doubtful, or, when these concerned well known clinical taxa described without deposition of type material, were neotypified. Neotypes (NT) in the present article have a single CBS (Centraalbureau voor Schimmelcultures) number, which refers to dried holotype material, or to metabolically inactivated samples under liquid nitrogen of which the original batch will remain unopened. In case the original holotype may not be interpretable, epitypes (ET) were indicated. If no type was indicated in the original protologue, but strains from the describing author(s) were available, these were listed as authentic (AUT). If none of these applies, but strains were used by authoritative authors, they were listed as reference strains. The latter two categories do not have official nomenclatural status.

Strains analyzed

Strains preserved in the reference collection of Centraalbureau voor Schimmelcultures (CBS-KNAW Fungal Biodiversity Centre) were used for the multilocus phylogenetic analysis of members of the family *Arthrodermataceae*. In total, 264 strains were analyzed. Strains were cultured on Sabouraud's glucose agar (SGA) plates using lyophilized, cryo-preserved or fresh mycelial material for inoculation. Most of the cultures were incubated for 7 to 14 days at the temperature of 24 °C, with some exceptions for very slow-growing species, while some others grew within a few days. The list of strains with their current and new taxon names are given in the supplementary Table ST1.

DNA extraction, PCR and sequencing

Genomic DNA was isolated from either preserved material or material harvested from living cultures. The DNA extraction was performed using MasterPure™ Yeast DNA Purification Kit from Epicentre. Five gene regions were amplified: ITS and LSU loci of the rDNA operon (29) and two protein coding genes. The universal fungal locus ITS1-5.8-ITS2 of the rDNA was amplified with ITS5 (30) and ITS4 (31) according to standard protocols (32). The D1-D2 region of LSU was amplified using primers LR0R and LR5 (33) according to conditions as for ITS except for a longer extension time (90 s). Partial ß-tubulin (*TUB*) was amplified with primers TUB2Fd and TUB4Fd (34). PCR had an annealing temperature of 58 °C for one min and elongation time of 70 s. Ribosomal protein *60S L10* was amplified with 60S-908R and 60-S506F (35).
All PCRs were done in 12.5 lL final PCR volume (CBS-KNAW barcoding lab protocol), using 2.5 lL of the DNA extract, 1.25 lL PCR buffer (Takara, Japan, incl. 2.5 mM $MgCl_2$), 1 lL dNTPs (1 mM stock; Takara, Japan), 0.6 lL dimethylsulfoxide (DMSO; Sigma, The Netherlands), forward-reverse primer 0.25 lL each (10 mM stock), 0.06 lL (5 U) Takara HS Taq polymerase, 7.19 lL MilliQ water (32, 36). PCR products were visualized on 1 % agarose gel. Positive PCR products were sequenced in cycle-sequencing reaction using ABI big dye terminator v.3.1 using only one quarter of the suggested volume (modified manufacturer's protocol). Bidirectional sequencing was performed in a capillary electrophoresis system (Life Technologies 3730XL DNA analyser). The obtained sequences were manually edited, and consensus sequences were stored in a Biolomics database (37).

Sequence alignment and phylogenetic analysis

Sequences were aligned with MAFFT v. 6.850b using default settings except for the 'genafpair' option (38). The datasets for the five loci were assembled in one multilocus dataset using sequence matrix software and deposited in Genbank. Alignments were compared manually and via the Gblocks server (http://molevol. cmima.csic.es/castresana) with stringency settings 'allow gaps positions within the final blocks' and 'do not allow many contiguous nonconserved positions'. For both ITS and multilocus dataset Maximum likelihood phylogeny was inferred using RAxML v. 8.0.0 employing GTRCAT model and 1000 bootstrap replicates. Bootstrap branch supports above 70 % are shown. One general and two more detailed multilocus single-genus trees are provided in summary (Figs. 3, 4, 5).

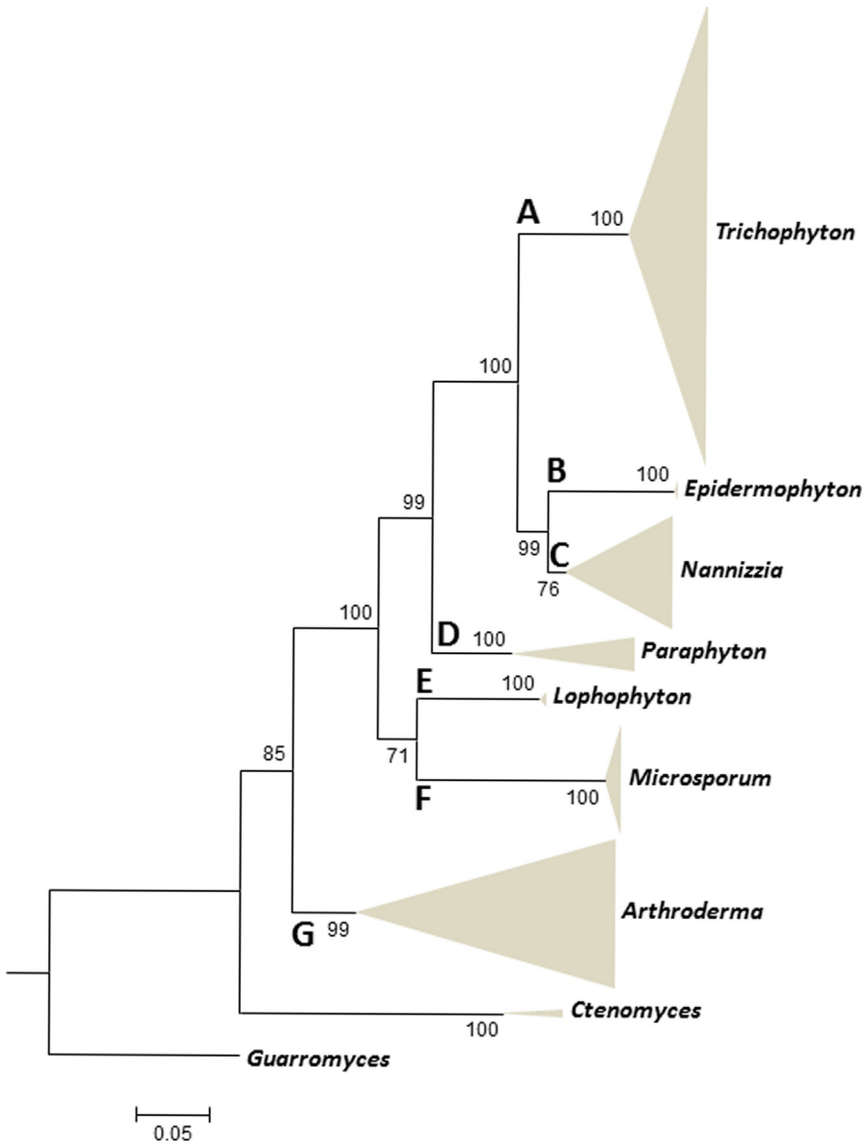

Fig. 3 Maximum likelihood phylogenetic tree (RAxML v.8.0.0) based on ITS and partial LSU, *TUB* and *60S L10* sequences of *Arthrodermataceae* species using GTRCAT as model, with 1000 bootstrap replications, shown when >70 %, where genera were collapsed. *Guarromyces ceretanicus* was selected as outgroup.

Results and Discussion

A phylogenetic trees were constructed for all species discussed in this paper using the ITS rDNA region only (data not shown) and the concatenated ITS, LSU, *TUB* and *60S L10* dataset shown in Fig. 3. Seven clades were distinguishable. The upper clade (A) in this figure comprised anthropophilic and zoophilic *Trichophyton* species. This clade is shown in more detail with multilocus data in Fig. 4. Four 100 % bootstrap-supported species or species series were recognizable: (A-1) *Trichophyton mentagrophytes* and related anthropophilic and zoophilic species including some strictly anthropophilic clonal offshoots, with *Trichophyton interdigitale* and *T. tonsurans* as most common species; (A-2) *Trichophyton benhamiae* series with *T. schoenleinii* and *T. verrucosum*; (A-3) The zoophilic species *Trichophyton bullosum*;


A-1
A-2
CBS 112192 Trichophyton tonsurans (horse)
CBS 338.37T Trichophyton immergens (skin)
CBS 318.31T Trichophyton floriforme
CBS 496.48NT Trichophyton tonsurans (scalp)
CBS 112195 Trichophyton tonsurans (horse)
CBS 109033 Trichophyton tonsurans (horse)
CBS 112189 Trichophyton tonsurans (human)
CBS 112190 Trichophyton tonsurans (horse)
CBS 112187 Trichophyton tonsurans (human)
CBS 112186 Trichophyton tonsurans (human)
CBS 112194 Trichophyton tonsurans (horse)
CBS 109034 Trichophyton tonsurans (horse)
CBS 112191 Trichophyton tonsurans (human
CBS 112193 Trichophyton tonsurans (horse)
CBS 182 76 Trichophyton tonsurans (horse)
Trichophyton tonsurans
CBS 100080T Trichophyton equinum var. autotrophicum (horse)
CBS 109036 Trichophyton equinum (skin)
CBS 270.66NT Trichophyton equinum (horse)
CBS 285.30NT Trichophyton areolatum
Trichophyton equinum
CBS 232.76 Trichophyton interdigitale (skin)
CBS 647.73T Trichophyton candelabrum (nail)
CBS 110.65 Trichophyton interdigitale (groin)
CBS 475.93T Trichophyton krajdenii (skin)
CBS 559.66 Trichophyton interdigitale (skin)
CBS 113880 Trichophyton interdigitale (nail)
CBS 425.63T Trichophyton batonrougei
CBS 449.74 Trichophyton interdigitale (skin)
CBS 428.63NT Trichophyton interdigitale (skin)
CBS 117723 Trichophyton interdigitale (skin)
CBS 124419 Trichophyton interdigitale
CBS 119447 Trichophyton interdigitale (scalp)
Trichophyton interdigitale
CBS 124425 Trichophyton mentagrophytes (cat)
CBS 124424 Trichophyton mentagrophytes (chamois)
CBS 124410 Trichophyton mentagrophytes (dog)
CBS 108.91 Trichophyton mentagrophytes
CBS 124404 Trichophyton mentagrophytes (rabbit)
CBS 124408 Trichophyton mentagrophytes (dog)
CBS 126.34T Trichophyton abyssinicum (skin)
CBS 124401 Trichophyton mentagrophytes (guinea pig)
CBS 117724 Trichophyton mentagrophytes (skin)
CBS 124421 Trichophyton mentagrophytes (rabbit)
CBS 124420 Trichophyton mentagrophytes (rabbit)
IHEM 4268NT Trichophyton mentagrophytes (skin)
CBS 646.73T Arthroderma vanbreuseghemii
CBS 120357 Trichophyton mentagrophytes (scalp)
CBS 120356 Trichophyton mentagrophytes (scalp)
CBS 120324 Trichophyton mentagrophytes (skin)
CBS 124415 Trichophyton mentagrophytes (cat)
CBS 124426 Trichophyton mentagrophytes (dog)
Trichophyton mentagrophytes
CBS 304.38T Trichophyton radicosum
CBS 158.66 Trichophyton quinckeanum (skin)
IHEM 13697NT Trichophyton quinckeanum (mouse)
CBS 318.56 Trichophyton quinckeanum (skin)
Trichophyton quickeanum
CBS 458.59NT Trichophyton schoenleinii (human)
CBS 118538 Trichophyton schoenleinii (scalp)
CBS 433.63 Trichophyton schoenleinii (scalp)
CBS 118539 Trichophyton schoenleinii (scalp)
Trichophyton schoenleinii
CBS 417.65T Trichophyton simii (poultry)
CBS 449.65ST Arthroderma simii (poultry)
CBS 448.65ST Arthroderma simii (poultry)
CBS 520.75 Trichophyton simii (macaca)
Trichophyton simii
CBS 624.66ST Arthroderma benhamiae (human)
CBS 623.66ST Arthroderma benhamiae
CBS 196.26NT Trichophyton concentricum (skin)
CBS 563.83 Trichophyton concentricum (skin)
CBS 448.61 Trichophyton concentricum (skin)
Trichophyton concentricum
CBS 112369 Trichophyton benhamiae (skin, from guinea pig)
CBS 112368 Trichophyton benhamiae (skin, from guinea pig)
CBS 112370 Trichophyton benhamiae (skin, from guinea pig)
CBS 934.73 Trichophyton benhamiae
CBS 809.72 Trichophyton benhamiae
CBS 280.83 Trichophyton benhamiae (skin)
CBS 120669 Trichophyton benhamiae (guinea pig)
CBS 112859 Trichophyton benhamiae (skin, from rabbit)
CBS 112857 Trichophyton benhamiae (skin, from guinea pig)
CBS 806.72 Trichophyton benhamiae (guinea pig)
Trichophyton benhamiae
CBS 511.73T Trichophyton erinacei (hedghog)
CBS 344.79 Trichophyton erinacei (skin)
CBS 474.76T Trichophyton proliferans (skin)
CBS 124411 Trichophyton erinacei (dog)
Trichophyton erinacei
CBS 282.82 Trichophyton verrucosum (cow)
CBS 130947 Trichophyton verrucosum (skin)
CBS 326.82 Trichophyton verrucosum (cow)
CBS 365.53NT Trichophyton verrucosum (cow)
CBS 130944 Trichophyton verrucosum (scalp)
CBS 130946 Trichophyton verrucosum (scalp)
CBS 161.66 Trichophyton verrucosum (skin)
Trichophyton verrucosum
CBS 220.25T Trichophyton eriotrephon (skin)
Trichophyton eriotrephon

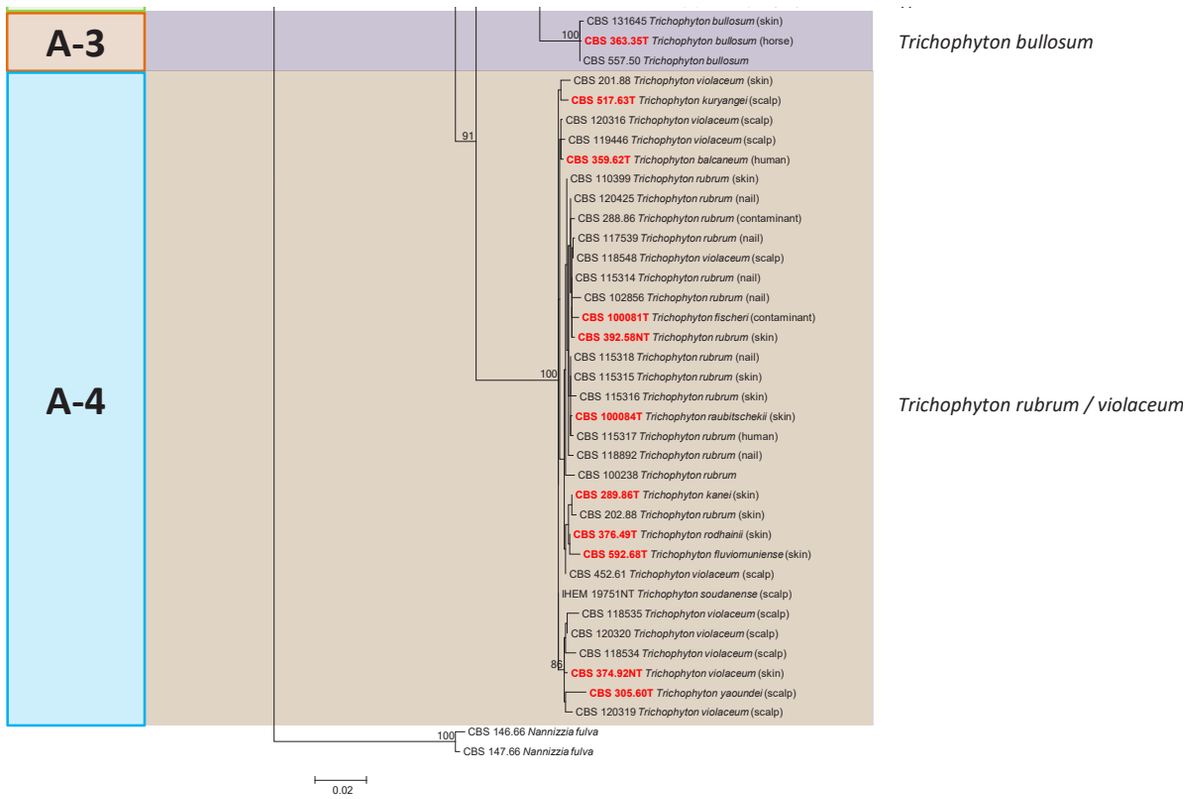

Figure. 4 Maximum likelihood phylogenetic tree (RAxML v.8.0.0) based on ITS and partial LSU, *TUB* and *60S L10* sequences of *Trichophyton* species using GTR + GI as model, with 1000 bootstrap replications, shown when >70 %. *Nannizzia fulva* was selected as outgroup

(A-4) *Trichophyton rubrum* series in which no individual species could be distinguished. The next, well-supported clade (B) in Fig. 3 contained a single species, *Epidermophyton floccosum*, which is paraphyletic to clade (C). Clade (C) contained zoophilic and geophilic species of which *Microsporum gypseum* was the most common one. Clades (D) and (E) were two groups of large conidial, heterothallic species. Clade (F) comprised the *Microsporum canis* series, which is shown in more detail with multilocus data in Fig. 5. Clade (G) was highly diverse, containing well-resolved geophilic species only, many of which are currently known under their *Arthroderma* teleomorph name because of heterothallic mating. The anamorphs were characterized by large, multi-celled, thick and rough-walled macroconidia and abundant microconidia.

Data were also generated for additional partial genes LSU, *60S L10*, and *TUB* (Figs. 4, 5). Clades (A) and (F), containing the great majority of species that are relevant in clinical and veterinary settings, were partially resolved. A number of classical species in medical and veterinary mycology proved to be indistinguishable, possibly due to the fact that the large number of SNPs overshadowed consistent differences. The application of the G blocks tool, reducing ambiguously aligned positions, led to inclusion of only 39 % of the original 830 positions in ITS and reduced the resolution between species. For this reason, we maintained manually aligned datasets and used additional phenotypic and ecological data for species delimitation. This did not always yield expected results; further detailed studies with mating tests remain necessary. In this study we differentiate ‘species series’, which are larger clusters of taxa which unite at the ITS level, and ‘species complexes’. Chen et al. (39) defined a complex as

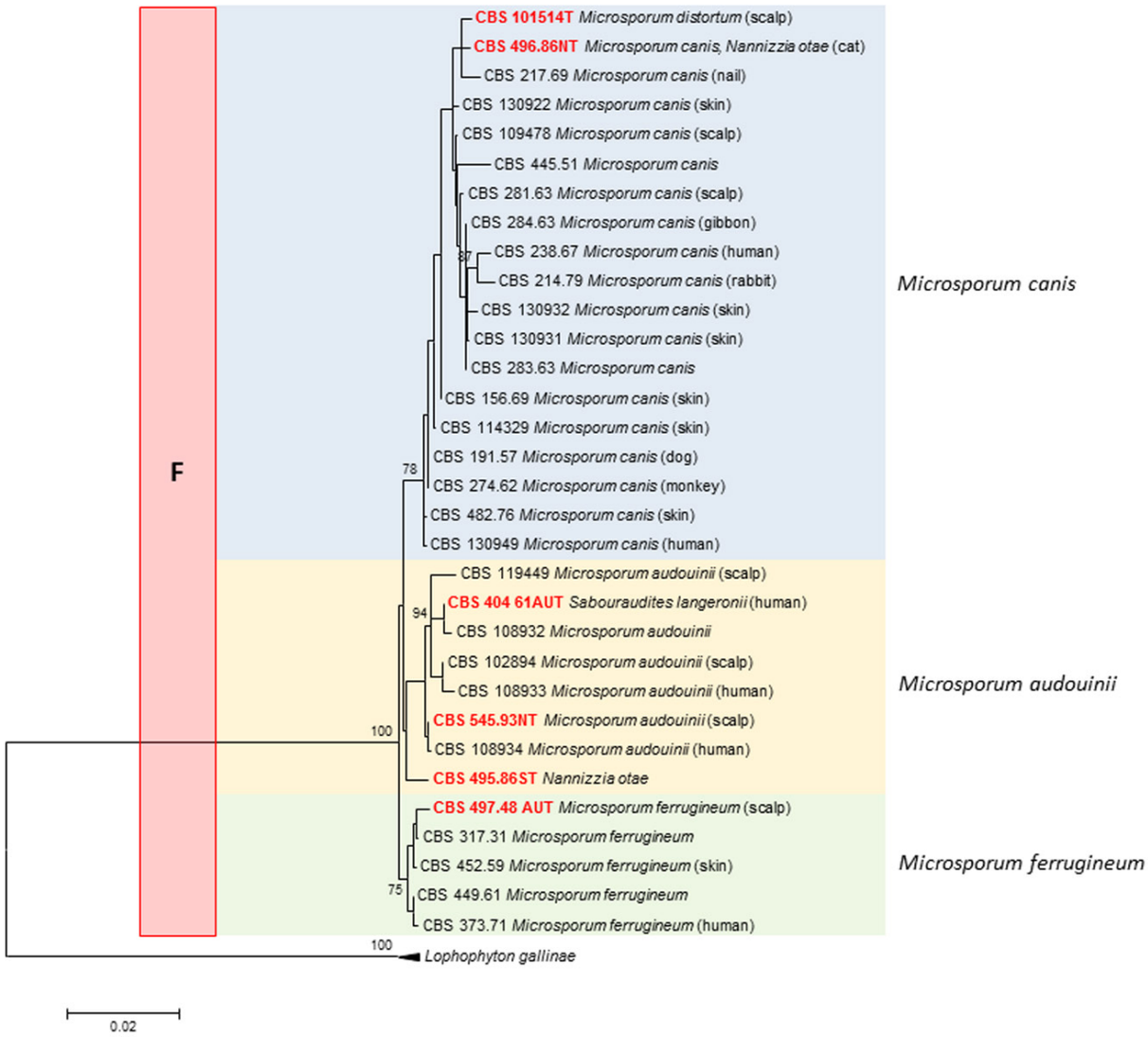

Figure. 5 Maximum likelihood phylogenetic tree (RAxML v.8.0.0) based on ITS and partial LSU, *TUB* and *60S L10* sequences of *Microsporum* species using T92 + G as model, with 1000 bootstrap replications, shown when >70 %. *Lophophyton gallinae* was selected as outgroup

a number of populations that are doubtfully distinct. In our species series, some of the taxa were unambiguously different when multilocus data were applied, while neighboring taxa could not properly be distinguished and thus might be regarded as species complexes. For precise species delimitation, data on natural hosts, virulence on non-optimal hosts, growth and sporulation, metabolite production and mating behavior are needed in addition to more detailed molecular studies. In the present overview, we prefer to be conservative in the maintenance of the number of species until more precise studies have proven exact borderlines of biological species and more understanding of host-specificity is acquired.

The species problem

In the *T. mentagrophytes* series (Clade A-1) in Fig. 4 showing a multilocus tree, *T. mentagrophytes* was close to *T. interdigitale*. The latter species was exclusively isolated

from humans, while *T. mentagrophytes* preponderantly originated from animals but also contained clinical strains. Trichophyton equinum could as yet not be distinguished from *T. tonsurans*. This touches on an essential question in medical mycology, as the species couples are known as zoophilic and anthropophilic, respectively, and a human infection by a zoophile is believed to be more inflammatory than when there is no host change. These questions cannot be solved in the present overview due to lack of clinical data of the strains examined. In the *T. benhamiae* series (Clade A-2), *Trichophyton benhamiae*, *T. concentricum*, *T. erinacei* and *T. verrucosum* could all be separated with multilocus data. *Trichophyton quinckeanum* is very close to *T. schoenleinii*. The *Trichophyton rubrum* complex (Clade A-4) showed some diversity, but this did not entirely match with observed differences in phenotype and clinical predilection. In clade (F), when analyzed with multilocus data (Fig. 5), *Microsporum canis*, *M. audouinii* and *M. ferrugineum* were difficult to distinguish, particularly because the (+) and (-) mating partners showed a mutual distance that spanned the diversity of nearly the entire genus. With distance, a gradational loss of sporulation is observed via an '*M. distortum* phenotype', concomitant with adaptation to the human host, which is in accordance with current species concepts.

A major taxonomic problem, frequently encountered in environmental fungi in general, is unexpected phylogenetic diversity of groups that previously seemed to be phenotypically monomorphic. Species with similar microscopic appearance sometimes even prove to belong to entirely different orders. Dermatophytes, in contrast, have consistently been found to belong to a single lineage, i.e., the family *Arthrodermataceae*. This shared phylogeny has been explained by their keratinophilic character, which is a rare property in the fungal kingdom. Evolution within the family shows a strong coherence with the animal hosts providing the keratin, as already noted in classical literature (40).

A second, current taxonomic problem is the molecular species concept. Almost everywhere in the fungal kingdom the number of molecular species appears to be much larger than what was earlier be recognized by conventional methods, see, for example, the fragmentation of *Aspergillus fumigatus* (41), *Candida parapsilosis* (42), or *Aureobasidium pullulans* (43). Again, the dermatophytes seem to be exceptional. In the course of 150 years medical mycology mainly focusing on Caucasians in Europe, and with a wide diversity of diseases from different body parts, an exhaustive amount of pheno- and genotypes has been investigated in numerous publications. About 10 species can be categorized as common anthropophilic dermatophytes on the Eurasian and North-American continents. However, in the Atlas of Clinical Fungi (7), 103 basionyms, with 242 synonymous names in total, have been extracted from the literature to describe these same ≈ 10 species. It appears that the diversity seen with conventional approaches is much higher than the existing genetic diversity. We may conclude that the anthropophilic and perhaps also the zoophilic dermatophytes have been over-classified. Similar phenomena of over-classification are apparent in other fungal groups of practical importance and which have therefore been studied *in extenso*. For example, *Rhizopus* species are easy to grow in culture, and their culturing has started immediately after Pasteur's time because of their role in fermentation processes of soy-based Asian foodstuffs. By 1920, 43 species were described in *Rhizopus microsporus* and *R. arrhizus*, which today are reduced to just two on molecular grounds (44, 45). Another example is the ubiquitous saprobe *Alternaria alternata*, where the large number of morphological taxa mainly distinguished previously on the basis of conidial shape and three-dimensional conidiophore branching patterns were reduced to synonymy on the basis of genomic data (46).

Phylogenetic overview

It may be concluded that the taxonomy of common anthropophilic dermatophytes is now mature enough to be stabilized at the benefit of clinical routine. Taxa that are recognized today are not likely to be subject to drastic change in the near future. Trees do not suffer from taxon sampling effects, and nomenclatural stability is within reach. Additional species on the human host are to be expected only among rare taxa, such as *Trichophyton eriotrephon*, degenerate and difficult to identify species, such as *Microsporum aenigmaticum*, species from geographically remote areas, such as *Trichophyton concentricum*, or from coincidental infections of otherwise zoo- or geophilic species. Particularly, the geophilic dermatophytes have insufficiently been studied compared to their large number of potential host animals and environmental habitats, and in these groups a larger number of taxonomic novelties can be expected, which however have limited clinical relevance.

The current main genera *Epidermophyton*, *Microsporum*, and *Trichophyton* in their classical circumscription are based on morphology of macroconidia. This corresponds only partly with phylogeny in that species fulfilling the morphological criteria of *Trichophyton* partly cluster in derived anthropophilic clades, and partly in ancestral clades of prevalently geophilic species (24). Consequently, a number of geophilic species which are phylogenetically remote from anthropophilic *Trichophyton* and hardly ever cause human infection are now included in routine identification panels (7). From ecological and clinical viewpoints, the difference between the two groups is immense, because anthropophilic species are considered to be real pathogens in that they have evolutionary advantage of being transmitted between human hosts, whereas an overwhelming number of geophilic species are opportunistic and are acquired from a natural habitat in the environment. The combination of such highly diverse fungi in a single genus is not optimal and might lead to inefficient use of hospital resources when pathogenic species have to be distinguished from numerous non-human taxa. Molecular phylogeny using 5 genes clearly separated the preponderantly geophilic species from the remainder, comprising several zoophilic and a preponderantly anthropophilic clade, which confirms previously published topologies based on ITS (6), *TEF1* (28) and *CAL* (47). Most zoophilic species compose clusters that are clearly separate from the preponderantly anthropophilic clades of *Trichophyton* and *Epidermophyton*. Now is the time to draw final conclusions and formulate the dermatophyte system in a modern sense, based on molecular phylogeny, supported by polyphasic data, and providing better tools for identification. This leads to a novel phylogenetic taxonomy and genus delimitation as outlined below. Main sets of criteria for species delimitation optimally should be based on the biological species concept, i.e., random mating with fertile progeny among members of the same species, and absence of mating between species. However, in microbiological practice, this criterion is often not easily applicable. Mating experiments and observation of fertile cleistothecia were particularly helpful to delineate species of the *M. gypseum* and of the *T. mentagrophytes* series (19, 20, 48–50). However, sexual reproduction is often not known because the conditions under which teleomorphs are produced are unknown, or perhaps they may not exist at all. Inter-sterile populations may exist within what we regard as a single species. In dermatophytes, preponderance of a single mating type which may have mating type associated properties may lead to asexual offshoots, explaining the clonal genetic composition of many species or other entities (51). An alternative approach is genealogical concordance, i.e., the biological species concept expressed in silico. In the present study, this approach was adopted using four genes: LSU, ITS, *60S*, and *TUB*. Different levels of resolution

of clades were obtained with these genes. Listing the number of clades supported by bootstrap values >80 %, we observe ITS>*TUB*>*60S*>LSU, yielding 44, 37, 32, and 17 clades, respectively (data not shown). For routine diagnostics, ITS is optimal, although for distinction of individual members of species complexes additional genes like TUB are necessary.

Once species have been delimited, the entities should be named according to the new rules of fungal nomenclature where Art. 59 of the ICBN regulating the pleomorphic naming system was abandoned. In principle the oldest name stands. From January 1, 2013, onwards, teleomorph names that are added later are considered as new combinations of the original basionym rather than as separate names. For older publications, the pleomorphic nomenclature still stand, in the sense that the different phases of the fungus are treated as facultative synonyms, even if they are introduced in the same paper and when based on the same type specimen. Often these types date back before 1958 since when explicit deposition was required (Art. 40 ICBN); in such cases the type of the teleomorph was selected as neotype of the species. In this way the currently accepted species is closely approached. The oldest, best known and widely used species names were mostly introduced even culture methods were available, and most of the nineteenth century names were based on clinical appearance only. Original materials are available of only a small selection of much younger taxa and synonyms. In order to maintain species names in current circumscriptions, widely used names are fixed by neotypes. In contrast, obsolete names for which no type materials are available are regarded as of doubtful identity and are thus permanently discarded.

Nomenclature

Clades (A–G) in Fig. 3 are judged to represent genera. Table 2 summarizes and evaluates all genera described in dermatophyte taxonomy since 1841, and Table 3 provides the distribution of extant type species of each of these genera over the phylogenetic tree of Fig. 3. The oldest legitimate generic names available for each of the clades are valid, reducing later names as synonyms. The only exception is clade A for which the name *Trichophyton* is preferred over *Achorion*; a proposal for conservation of the former name is being prepared. Below the genera and species attributed to them are listed.

Clade A: *Trichophyton*

Colonies mostly cottony, white to yellowish, with a cream-colored, brown, red, violet colony reverse. Hyphae thin-walled, hyaline. Thallic macroconidia and microconidia, if present, terminally on or alongside undifferentiated hyphae. Macroconidia, 2- or multi-celled, thin- and smooth-walled, hyaline, cylindrical, or clavate to cigar-shaped. Microconidia thinand smooth-walled, hyaline, 1-celled, ovoidal, pyriform to clavate. Sexual state sometimes present after mating, arthroderma-like.

Type species: *Trichophyton tonsurans* Malmsten.

1. *Trichophyton benhamiae* (Ajello & Cheng) Gräser & de Hoog, **comb. nov**.

Basionym: *Arthroderma benhamiae* Ajello & Cheng, Sabouraudia 5: 232, 1967. Holotype NCDC B765d; type strain cross of CBS 623.66 = ATCC 16781 = CDC X797 (MT+) x CBS 624.66 = ATCC 16782 = CDC X798(MT-), UK, L. Ajello. Zoophilic species, mainly on guinea pigs (52), occasionally other animals (53). A white and a yellow phenotype are known, the yellow genotype containing MT- strains only (20); a hybridization depression is noted with

the remaining lineages. Contet-Andonneau & Leyer (54) invalidly introduced *Trichophyton erinacei var. porcellae* (without indication of type specimen, Art. 52 (ICBN) matching the yellow phenotype. Note that with multilocus sequencing the mating types deviate slightly from remaining strains (Fig. 4).

2. ***Trichophyton bullosum*** Lebasque, Champ. Teign. Cheval Bovidés p. 53, 1933.
Type strain: CBS 363.35, from horse, France. Zoophilic species (55).

3. ***Trichophyton concentricum*** Blanchard, in Bouchard, Traité Path. Gén. 2: 916, 1896 ≡ *Lepidophyton concentricum* (Blanchard) Gedoelst, Champ. Paras. Homme Anim. Domest. p. 147, 1902 ≡ *Aspergillus concentricum* (Blanchard) Castellani, Trans. Int.
Derm. Congr. 6: 671, 1907 ≡ *Endodermophyton concentricum* (Blanchard) Castellani & Chalmers, Man. Trop. Med. p. 610, 1910 ≡ *Oospora concentrica* (Blanchard) Hanawa & Nagai, Jpn. J. Derm. Urol., Suppl., p. 47, 1917 ≡ *Achorion concentricum* (Blanchard) Guiart & Grigoraki, Lyon Méd. 141: 377, 1928 ≡ *Mycoderma concentricum* (Blanchard) Vuillemin, C. R. Hebd. Séanc. Acad. Sci., Paris 89: 405, 1929. Neotype strain: CBS 196.26 = IFO 5926, A. Castellani, 1926. Anthropophilic species causing tinea imbricata in Polynesia (56).

4. ***Trichophyton equinum*** Gedoelst, Champ. Paras. Homme p. 88, 1902 ≡ *Ectotrichophyton equinum* (Gedoelst) Castellani & Chalmers, Man. Trop. Med., ed. 3, p. 1007, 1919 ≡ *Megatrichophyton equinum* (Gedoelst) Neveu-Lemaire, Précis Parasitol. Hum., ed. 5, p. 54, 1921 ≡ *Ctenomyces equinus* (Gedoelst) Nannizzi, Tratt. Micopat. Um. 4: 144, 1934. Neotype designated here: CBS 270.66, from horse, USA, L.K. George. Zoophilic species, but, at least based on DNA sequences, doubtfully distinct from *T. tonsurans* which is generally regarded as anthropophilic. The species are phenotypically distinguished by brown colonies and larger microconidia in *T. tonsurans*.
Fac. syn.: *Trichophyton areolatum* Negroni, Annls Parasit. Hum. Comp. 7: 438, 1929. Type strain: CBS 285.30, Argentina, P. Negroni.
Fac. syn.: *Trichophyton equinum* Gedoelst var. *autotrophicum* J.M.B. Smith, Jolly, Georg & Connole, Sabouraudia 6: 297, 1968. Type strain: CBS 100080 = ATCC 22443 = IMI 133568, from horse, New Zealand.

5. ***Trichophyton eriotrephon*** Papegaaij, Nederl. Tijdschr. Geneesk. 69: 885, 1925. Type strain: CBS 220.25, from ringworm of female patient, The Netherlands, J. Papegaaij, 1925.

6. ***Trichophyton erinacei*** (J.M.B. Smith & Marbles) Quaife; *Trichophyton mentagrophytes* (Robin) Blanchard var. erinacei J.M.B. Smith & Marbles, Sabouraudia 3: 9, 1963 ≡ *Trichophyton erinacei* (J.M.B. Smith & Marbles) Quaife, J. Clin. Path. 19: 178, 1966 ≡ *Arthroderma benhamiae* Ajello & Cheng var. erinacei (J.M.B. Smith & Marbles) Takashio, Bull. Soc. Fr. Mycol. Méd. 4: 47, 1975. Holotype: IMI 101051; type strain: CBS 511.73 = ATCC 28443 = IMI 101051 = NCPF 375, from hedgehog, New Zealand.

7. ***Trichophyton interdigitale*** Priestley, Med. J. Aust. 4: 475, 1917 ≡ *Sabouraudites interdigitalis* (Priestley) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 328, 1923 ≡ *Epidermophyton interdigitale* (Priestley) MacCarthy, Archs Derm. Syph. 6: 24, 1925 ≡ *Trichophyton mentagrophytes* (Robin) Blanchard var. *interdigitale* (Priestley) Moraes, Anais Bras. Derm. Sif. 25: 230, 1950 ≡ *Kaufmannwolfia interdigitalis* (Priestley) Galgóczy & Novák, in Bakács, Az Orsz. Köz. Intöz. Mök. p. 224, 1962 ≡ *Microides interdigitalis* (Priestley) De Vroey, Ann. Soc. Belg. Méd. Trop. 50: 25, 1970. Neotype strain: CBS 428.63, from human foot, The Netherlands, M. Bruining (6). Anthropophilic species, almost exclusively found in non-inflammatory tinea unguium and tinea pedis. The species may be regarded as a clonal offshoot of *T. mentagrophytes*. The position of CBS 119447 requires further study.
Fac. syn.: *Trichophyton batonrougei* Castellani, J. Trop. Med. Hyg. 42: 373, 1939 ≡ *Trichophyton*

mentagrophytes (Robin) Blanchard var. *batonrougei* (Castellani) de Vries & Cormane, Ned. Tijdschr. Geneeskd. 109: 1426, 1965. Type strain: CBS 425.63, A. Castellani.

Fac. syn.: *Trichophyton candelabrum* Listemann, Castellania 1: 53, 1973. Type strain: CBS 647.73, from human toenail, Germany, H. Listemann.

Fac. syn.: *Trichophyton krajdenii* J. Kane, J.A. Scott & Summerbell, Mycotaxon 45: 309, 1992. Type: CBS 475.93 = UAMH 3244, from human skin, Canada, J. Kane.

Fac. syn.: *Trichophyton radicosum* Catanei, Arch. Inst. Pasteur Algér. 15: 267, 1937. Type strain: CBS 304.38, A. Catanei, May 1938. Note that in the multilocus tree (Fig. 4), the position of the type strain is unresolved.

8. ***Trichophyton mentagrophytes*** (Robin) Blanchard; *Microsporum mentagrophytes* Robin, Hist. Nat. Vég. Paras. Homme Anim. p. 430, 1853 ≡ *Sporotrichum mentagrophytes* (Robin) Saccardo, Syll. Fung. 4: 100, 1886 ≡ *Trichophyton mentagrophytes* (Robin) Blanchard, Traité Pathol. Gén. 2: 811, 1896 ≡ *Ectotrichophyton mentagrophytes* (Robin) Castellani & Chalmers, Man. Trop. Med., ed. 3. p. 1005, 1919 ≡ *Ctenomyces mentagrophytes* (Robin) Langeron & Milochevitch, Annls Parasit. Hum. Comp. 8: 484, 1930 ≡ *Spiralia mentagrophytes* (Robin) Grigoraki, C. R. Séanc. Soc. Biol. 109: 186, 1932 ≡ *Microides mentagrophytes* (Robin) De Vroey, Ann. Soc. Belg. Méd. Trop. 50: 25, 1970. As neotype, CBS 318.56 has been selected (6), but this was disputed by several authors (57–59). Chollet et al. (60) convincingly showed that the original case of C. Robin concerned a human tinea barbae, a disorder generally ascribed to zoophilic species. Isolates of this species show some ITS diversity but are either from animals or from patients with inflammatory dermatophytoses indicating an animal origin; reservoirs are hunting cats, dogs (52), mice (19) and horses (61). Isolates are able to mate with *Arthroderma* strains (50). An alternative **neotype designated herewith** IHEM 4268, from tinea corporis of human face, Brussels, Belgium, which is more in accordance with the protologue. Note that until recently a distinction was made between anthropophilic and zoophilic strains of *T. mentagrophytes* (62). Truly anthropophilic, low-inflammatory strains correspond with the clonal offshoot *T. interdigitale*, while more inflammatory human infections by zoophilic strains match with *T. mentagrophytes s. str*.

Fac. syn.: *Bodinia abyssinica* Agostini, Atti Ist. Bot. Lab. Crittogam. Univ. Pavia, Ser. 4, 2: 123, 1931 ≡ *Trichophyton abyssinicum* (Agostini) Nannizzi, Tratt Micopat. Um. 4: 174, 1934 ≡ *Favotrichophyton abyssinicum* (Agostini) C.W. Dodge, Med. Mycol. p. 517, 1935. Type strain: CBS 126.34, from human skin, G. Pollacci, 1934.

Fac. syn.: *Arthroderma vanbreuseghemii* Takashio, Ann. Soc. Belg. Méd. Trop. 53: 547, 1973. Type strain: CBS 646.73 = ATCC 28145 = CECT 2900 = IHEM 3299 = NCPF 452 (MT+), M. Takashio.

9. ***Trichophyton quinckeanum*** (Zopf) MacLeod & Münde; *Oidium quinckeanum* Zopf, Die Pilze p. 481, 1890 ≡ *Achorion quinckeanum* (Zopf) Bodin, Archs Parasit. 5: 5–30, 1902 ≡ *Sabouraudites quinckeanus* (Zopf) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 328, 1923 ≡ *Closteroaleuriospora quinckeana* (Zopf) Grigorakis, Annls Sci. Nat., Bot., Sér. 10, 12: 412, 1925 ≡ *Microsporum quinckeanum* (Zopf) Guiart & Grigorakis, Lyon Méd. 141: 377, 1928 ≡ *Trichophyton quinckeanum* (Zopf) MacLeod & Münde, Pract. Handb. Skin p. 361, 1940 ≡ *Trichophyton gypseum* Bodin var. *quinckeanum* (Zopf) Frágner, Česká Mykol. 10: 106, 1956 ≡ *Trichophyton mentagrophytes* (Robin) Blanchard var. *quinckeanum* (Zopf) J.M.B. Smith & Austwick, in Cotchin & Roe, Path. Lab. Rats Mice p. 684, 1967. **Neotype designated herewith**: IHEM 13697 = RV 32626 = CDC X393, from mouse favus, Philadelphia, USA, H. Blank. Zoophilic species causing favus on mice (59). Member of the *T. mentagrophytes* series.

10. ***Trichophyton rubrum*** (Castellani) Semon; *Epidermophyton rubrum* Castellani, Phil. J. Sci. 5: 203, 1910 ≡ *Trichophyton rubrum* (Castellani) Semon, Br. J. Derm. Syph. 34: 398, 1922 ≡ *Sabouraudites ruber* Ota & Langeron, Annls Parasit. Hum. Comp. 1: 328, 1923: *Sabouraudiella rubra* (Castellani) Boedijn, Mycopath. Mycol. Appl. 6: 125, 1951. Neotype strain: CBS 392.58, from human, The Netherlands, H. Esseveld. Anthropophilic species, the most prevalent recognized infectious agent in onychomycoses (tinea unguium) and tinea pedis, also causing tinea cruris and tinea corporis; it has a global distribution. *Trichophyton megninii* Blanchard is often listed as a synonym of *Trichophyton rubrum*, but no type material is known to exist.
Fac. syn.: *Trichophyton balcaneum* Castellani, J. Trop. Med. Hyg. 22: 174, 1919. Type strain: CBS 359.62, from human,USA,T. Benedek. The identity of this strain is uncertain and should be re-investigated.
Fac. syn.: *Trichophyton rodhainii* Vanbreuseghem, Annls Parasit. Hum. Comp. 24: 244, 1949 *Trichophyton rubrum* Castellani var. *rodhainii* (Vanbreuseghem) Armijo & Lachapelle, Annls Derm. Vénéréol. 108: 990, 1981. Type strain: CBS 376.49, from tinea cruris of Caucasian in Congo, R. Vanbreuseghem.
Fac. syn.: *Trichophyton fluviomuniense* Pereiro Miguens, Sabouraudia 6: 315, 1968. Type strain: CBS 592.68 = ATCC 22402, from human skin, Guinea, M. Pereiro Miguens.
Fac. syn.: *Trichophyton fischeri* Kane, Sabouraudia 15: 239, 1977. Type strain: CBS 100081 = ATCC 32871 = IMI 213848, culture contaminant, Toronto, Canada.
Fac. syn.: *Trichophyton raubitschekii* Kane, Salkin, Weitzman & Smitka, Mycotaxon13: 260, 1981 ≡ *Trichophyton rubrum* (Castellani) Semon var. *raubitschekii* (Kane, Salkin, Weitzman & Smitka) Brasch, Mycoses 50, Suppl. 2: 2, 2007. Type strain: CBS 100084 = ATCC 42631, from human, Canada, J. Kane.
Fac. syn.: *Trichophyton kanei* Summerbell, Mycotaxon 28: 511, 1987. Type strain: CBS 289.86 = ATCC 62345 = TRTC 50887, from human skin, Canada, R.C. Summerbell.
11. ***Trichophyton schoenleinii*** (Lebert) Nannizzi; *Oidium schoenleinii* Lebert, Physiol. Path. 2: 490, 1845 ≡ *Achorion schoenleinii* (Lebert) Remak, Diagn. Pathog. Unters. p. 13, 1845 ≡ *Schoenleinium achorion* Johan-Olsen, Zentbl. Bakt. Parasitkde, Abt. 2, 3: 276, 1897 (name change) ≡ *Grubyella schoenleinii* (Lebert) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 320, 1923 ≡ *Arthrosporia schoenleinii* (Lebert) Grigoraki, Annls Sci. Nat., Bot., Sér. 7: 414, 1925 ≡ *Sporotrichum schoenleinii* (Lebert) Saccardo, in Vuillemin, Champ. Paras. Myc. Homme p. 69, 1931 ≡ *Trichophyton schoenleinii* (Lebert) Nannizzi, Tratt. Micopat. Um. 4: 198, 1934. Neotype designated herewith: CBS 458.59, from human scalp, The Netherlands, F.H. Oswald. Anthropophilic species.
12. ***Trichophyton simii*** (Pinoy) Stockdale, MacKenzie & Austwick; *Epidermophyton simii* Pinoy, C. R. Soc. Biol.72: 59, 1912 ≡ *Pinoyella simii* (Pinoy) Castellani & Chalmers, Man. Trop. Med., ed. 3, p. 1023, 1919 ≡ *Arthroderma simii* (Pinoy) Stockdale, MacKenzie & Austwick, Sabouraudia 4: 113, 1965 ≡ *Trichophyton simii* (Pinoy) Stockdale, MacKenzie & Austwick, Sabouraudia 4: 114, 1965. The type material of *E. simii* is not known to be preserved. A teleomorph was introduced by Stockdale et al. (63), which is here taken as a new combination and is considered to be representative for the species. Holotype IMI 98944, authentic strains: CBS 417.65 = ATCC 16448 = IHEM 4420 = IMI 101695 = NCPF 394 (MT-), CBS 448.65 = ATCC 16447 = IHEM 4421 = IMI 101693 = NCPF 494 (MT+), CBS 449.65 = IMI 101694 = NCPF 393(MT+), all from poultry, India, C.O. Dawson. Zoophilic species.
13. ***Trichophyton soudanense*** Joyeux, C. R. Seanc. Soc. Biol. 73: 15, 1912 ≡ *Langeronia soudanensis* (Joyeux) Vanbreuseghem, Ann. Soc. Belg. Méd. Trop. 30: 888, 1950. Neotype

designated herewith: IHEM 19751 = RV 44663, from tinea capitis, Lomé, Togo, Tchalim, 1988. Anthropophilic species, very close to, perhaps even indistinguishable from *T. violaceum*, both species causing tinea capitis in northern Africa. More detailed studies are needed to establish species borderlines.

14. ***Trichophyton tonsurans*** Malmsten, harskärende Mägel. Bidrag till utredande af de sjukdomar, som valla harets affall. Stockholm, gr. 8, 1845; Arch. Anat. Physiol. Wiss. Med. 1848: 14, 1848 ≡ *Trichomyces tonsurans* (Malmsten) Malmsten, Arch. Anat. Physiol. Wiss. Med. 1848: 14, 1848 ≡ *Oidium tonsurans* (Malmsten) Zopf, Die Pilze p. 482, 1890. Neotype strain: CBS 496.48, from human scalp, France, M. Rivalier. Anthropophilic species (6).

Fac. syn.: *Trichophyton floriforme* Beintema, Arch. Dermatol. 169: 575, 1934. Type strain: CBS 318.31, K. Beintema.

Fac. syn.: *Trichophyton immergens* Milochevitch, C. R. Hebd. Séanc. Acad. Sci., Paris 124: 469, 1937. Type strain: CBS 338.37, from human glabrous skin, Serbia, S. Milochevitch.

15. ***Trichophyton verrucosum*** Bodin, Champ. Paras. Homme p. 121, 1902 ≡ *Ectotrichophyton verrucosum* (Bodin) Castellani & Chalmers, Man. Trop. Med., ed. 3, p. 1003, 1919: *Favotrichophyton verrucosum* (Bodin) Neveu-Lemaire, Précis Parasitol. Hum., ed. 5, p. 55, 1921. **Neotype designated herewith**: CBS 365.53, from cow, F. Blank. Zoophilic species on cattle.

16. ***Trichophyton violaceum*** Sabouraud, in Bodin, Champ. Paras. Homme p. 113, 1902 ≡ *Achorion violaceum* (Sabouraud) Bloch, Derm. 18: 815, 1911 ≡ *Sabouraudites violaceum* (Sabouraud) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 328, 1923 ≡ *Bodinia violacea* (Sabouraud) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 329, 1923 ≡ *Arthrosporia violacea* (Sabouraud) Grigoraki, Annls Sci. Nat., Bot., Sér. 10, 7: 414, 1925 ≡ *Favotrichophyton violaceum* (Sabouraud) C.W. Dodge, Med. Mycol. p. 523, 1935. Neotype strain: CBS 374.92, from human, The Netherlands, C.S. Tan (24). Anthropophilic species. Note that molecularly the species cannot be distinguished from T. rubrum (Fig. 4). Strains from human scalp generate *T. violaceum* phenotypes, so probably mutations in the pentaketide pathway interfering with the production of pigmented secondary metabolites are concerned.

Fac. syn.: *Trichophyton yaoundei* Cochet & DobyDubois, Annls Parasit. Hum. Comp. 32: 585, 1957. Type strain: CBS 305.60, G. Cochet, November 1960.

Fac. syn.: *Trichophyton kuryangei* Vanbreuseghem & Rosenthal, Annls Parasit. Hum. Comp. 36: 802, 1961. Type strain: CBS 517.63 = RV 8289, from tinea capitis of black infant, Kuryange, Usumbura Province, Ruanda Burundi, R. Vanbreuseghem.

Clade B: *Epidermophyton*

Colonies cottony, white to yellowish, with a cream-colored or brownish colony reverse. Hyphae thinwalled, hyaline. Thallic macroconidia terminally on or alongside undifferentiated hyphae, multi-celled, thinand smooth- or rough-walled, hyaline, cigar-shaped.

Microconidia absent. Sexual state unknown.

Type species: *Acrothecium floccosum* Harz.

1. ***Epidermophyton floccosum*** (Harz) Langeron & Milochevitch; *Acrothecium floccosum* Harz, Bull. Soc. Imp. Nat. Moscou 44: 124, 1871 ≡ *Blastotrichum floccosum* (Harz) Belese & Voglino, Add. Syll. Nr. 3604, 1886 ≡ *Dactylium floccosum* (Harz) Sartory, Champ. Paras. Homme Anim. p. 871, 1923 ≡ *Epidermophyton floccosum* (Harz) Langeron & Milochevitch, Annls Parasit. Hum. Comp. 8: 495, 1930 ≡ *Epidermomyces floccosus* (Harz) Loeffler, Mykosen

26: 446, 1983. **Neotype designated herewith**: CBS 230.76, from human, R.A. Zappey. Anthropophilic species.

Clade C: *Nannizzia*

Colonies mostly cottony to powdery, whitish to brown, with a cream-colored, brown or red. Hyphae thin-walled, hyaline. Thallic macroconidia and microconidia, if present, arranged in orthotropically arranged hyphal systems. Macroconidia, 2- or multicelled, thin- and smooth- or rough-walled, hyaline, cylindrical, or clavate to cigar-shaped. Microconidia thin- and smooth-walled, hyaline, 1- celled, ovoidal, pyriform to clavate. Sexual state commonly present after mating, arthroderma-like.
Type species: *Nannizzia incurvata* Stockdale.

1. ***Nannizzia aenigmatica*** (Hubka, Dobiášová & Kolařík) Gräser & de Hoog, **comb. nov**. Basionym: *Microsporum aenigmaticum* Hubka, Dobia´sˇova´ & Kolařík, Med. Mycol. 52: 389, 2014. Holotype: PRM 922698, type strain: CBS 134549 = CCF4608, skin of 46-year-old female,
Czech Republic, Ostrava, S. Dobiášová

Nannizzia corniculata Takashio & De Vroey, Mycotaxon 14: 384, 1982 ≡ *Arthroderma corniculatum* (Takashio & De Vroey) Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 513, 1986. Holotype: CBS-H 7400; type culture: CBS 364.81 = ATCC 46541 = IHEM 4409, from soil, Somalia.

2. ***Nannizzia duboisii*** (Vanbreuseghem) Gräser & de Hoog, **comb. nov**.
Basionym: *Sabouraudites duboisii* Vanbreuseghem, Annls Parasit. Hum. Comp. 24: 254, 1949 ≡ *Microsporum duboisii* (Vanbreuseghem) Ciferri, Man. Mic. Med., ed. 2: 414, 1960. Type strain: CBS 349.49, from human, Zaire, R. Vanbreuseghem.

3. ***Nannizzia fulva*** (Uriburu) Stockdale; *Microsporum fulvum* Uriburu, Argent. Med. 7, 1909 ≡ *Sabouraudites fulvus* (Uriburu) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 329, 1923 ≡ *Closterosporia fulva* (Uriburu) Grigoraki, Annls Sci. Nat., Bot. Sér. 10, 7: 411, 1925 ≡ *Nannizzia fulva* (Uriburu) Stockdale, Sabouraudia 3: 120, 1963 ≡ *Nannizzia gypsea* (Uriburu) Stockdale var. *fulva* (Uriburu) Apinis, Mycol. Pap. 96: 33, 1964 ≡ *Arthroderma fulvum* (Uriburu) Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 513, 1986. Holotype: IMI 10065, type strain: CBS 287.55, from human, Argentina, E. Rivalier. Types of teleomorph: CBS 168.64 = ATCC 16446 = IHEM 3296 = IMI 086180 = NCPF 391 (MT-) 9 ATCC 16445 = IHEM 3292 = IMI 086179 = NCPF 390 (MT+), both from soil, Hungary, S. Szathmary. Geophilic species.
Fac. syn.: *Keratinomyces longifusus* Flórián & Galgóczy, Mycopath. Mycol. Appl. 24: 76, 1964. Type strain: CBS 243.64 = ATCC 22397, from human, Hungary, E. Flórián, May 1964.
Fac. syn.: *Microsporum boullardii* Dominik & Majchrowicz, Ekol. Polska, Ser. A, 13: 426, 1965. Type strain: CBS 599.66 = ATCC 22399, from soil, Guinea, T. Dominik.
Fac. syn.: *Favomicrosporon pinettii* Benedek, Mycopath. Mycol. Appl. 31: 111, 1967. Authentic strains: CBS 146.66 = ATCC 16482, CBS 147.66 = ATCC 16481, T. Benedek.
Fac. syn.: *Microsporum ripariae* Hubálek & Rush-Munro, Sabouraudia 11: 288, 1973. Type strain: CBS 529.71 = ATCC 28005, from sand martin swallow (*Riparia riparia*), Czechia, Z. Hubálek.

4. ***Nannizzia gypsea*** (Nannizzi) Stockdale; *Gymnoascus gypseus* Nannizzi, Atti Accad. Fisioscr. Siena Med.-Fis. 2: 93, 1927 (*non Trichophyton gypseum* Bodin, Champ. Paras. Homme p. 115, 1902) ≡ *Nannizzia gypsea* (Nannizzi) Stockdale, Sabouraudia 3: 119, 1964 ≡

Arthroderma gypseum (Nannizzi) Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 514, 1986. Neotype strain: CBS 258.61 = IMI 80558, from soil, Australia, D.M. Griffin, Nov. 1961. Geophilic species.
Fac. syn.: *Microsporum appendiculatum* Bhat & Miriam, in Miriam & Bhat, Kavaka 25: 93, 1998. Holotype: GUFH 010, India, Goa University campus, herbarium specimen on decomposing goat dung, J. Miriam, 1996. Sharma et al. (64) showed that strains with appendiculate conidia were genetically identical to *M. gypseum*.
Fac. syn.: *Microsporum gypseum* (Bodin) Guiart & Grigoraki var. *vinosum* Gordon & Lusick, Archs Derm. 91: 562, 1965. Type strain: CBS 100.64 = ATCC 16428, from human, USA, 1964, M.A. Gordon.

5. ***Nannizzia incurvata*** Stockdale, Sabouraudia 1: 46, 1961 ≡ *Nannizzia gypsea* (Nannizzi) Stockdale var. *incurvata* (Stockdale) Apinis, Mycol. Pap. 96: 32, 1964 ≡ *Arthroderma incurvatum* (Stockdale) Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 514, 1986 ≡ *Microsporum incurvatum* (Stockdale) P.-L. Sun & Y.-M. Ju, Med. Mycol. 52: 280, 2014. Holotype: dried culture IMI 82777, type strain: CBS 174.64 = IMI 82777 = NCPF 236, from human skin, UK, P.M. Stockdale. Geophilic species, although also human infections occur. Stockdale (11) reported production of ascocarps using human-derived strains only.

6. ***Nannizzia nana*** (Fuentes) Gräser & de Hoog, **comb. nov**.
Basionym: *Microsporum gypseum* (Bodin) Guiart & Grigoraki var. *nanum* Fuentes, Aboulafia & Vidal, J. Invest. Derm. 23: 56, 1954 (invalid) ≡ *Microsporum nanum* Fuentes, Aboulafia & Vidal ex Fuentes, Mycologia 48: 614, 1956. Type strain: CBS 314.54 = ATCC 11832, from kerion of human scalp, C.A. Fuentes, June 1954. Zoophilic species on pigs; human inflammatory infections occur.
Fac. syn.: *Nannizzia obtusa* Dawson & Gentles, Sabouraudia 1: 56, 1961 ≡ *Arthroderma obtusum* (Dawson & Gentles) Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 514, 1986. Type: crossing of strains IMI 117073 (MT+) x IMI 117064 (MT-) (mating strains CBS 321.61, CBS 322.61), from human patient, C.O. Dawson and J.C. Gentles.

7. ***Nannizzia persicolor*** (Sabouraud) Stockdale; *Trichophyton persicolor* Sabouraud, Malad. Cuir Chev. 3: 632, 1910 ≡ *Ectotrichophyton persicolor* (Sabouraud) Castellani & Chalmers, Man. Trop. Med. p. 1005, 1918 ≡ *Sabouraudites persicolor* (Sabouraud) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 329, 1923 ≡ *Closteroaleuriosporia persicolor* (Sabouraud) Grigorakis, Annls Sci. Nat., Bot., Sér. 10, 7: 412, 1925 ≡ *Microsporum persicolor* (Sabouraud) Guiart & Grigorakis, Lyon Méd. 141: 377, 1928 ≡ *Ctenomyces persicolor* (Sabouraud) Nannizzi, Tratt. Micopat. Um. 4: 154, 1934 ≡ *Epidermophyton persicolor* (Sabouraud) C.W. Dodge, Med. Mycol. p. 486, 1935 ≡ *Langeronites persicolor* (Sabouraud) Ansel 1957 ≡ *Trichophyton mentagrophytes* (Robin) Blanchard var. *persicolor* (Sabouraud) Ueckert, Zentbl. Bakt. Parasitkde, Abt. 1, 176: 127, 1959 ≡ *Nannizzia persicolor* (Sabouraud) Stockdale, Sabouraudia 5: 357, 1967 ≡ *Microides persicolor* (Sabouraud) De Vroey, Ann. Soc. Belg. Méd. Trop. 50: 25, 1970 ≡ *Arthroderma persicolor* (Sabouraud) Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 514, 1986. The original material of Sabouraud is not known to be preserved. The name is defined by the teleomorph described by Stockdale (65) which is here taken to be meant as a new combination. The respective dried material is therefore a **neotype designated herewith**: IMI 126886, cross of living strains IMI 117073 (MT+), from bank vole, UK 9 IMI 117064 (MT-), from shrew, UK, M.E. English. Zoophilic species.
Fac. syn.: *Nannizzia quinckeani* Balabanov & Schick, Derm. Venereol. 9: 35, 1970. Type strain: CBS 871.70, from human skin, Bulgaria, V.A. Balabanov.

8. ***Nannizzia praecox*** (Rivalier ex A.A. Padhye, Ajello & McGinnis) Gräser & de Hoog, **comb. nov**.
Basionym: *Sabouraudites praecox* Rivalier, Annls Inst. Pasteur 86: 276, 1954 (invalid) ≡ *Microsporum praecox* (Rivalier) Rivalier, Bull. Soc. Fr. Mycol. Méd. 7: 297, 1978 (invalid) ≡ *Microsporum praecox* Rivalier ex Padhye, Ajello & McGinnis, in Padhye, Detweiler, Frumkin, Bulmer, Ajello & McGinnis, J. Med. Vet. Mycol. 27: 316, 1989. Holotype CDC B-4819D; authentic strain CBS 288.55, from human, E. Rivalier.

Clade D: *Paraphyton* Gräser Dukik & de Hoog, **gen. nov**

Colonies mostly granular, brownish, with a brown colony reverse. Hyphae thin-walled, hyaline. Thallic macroconidia and microconidia, if present, arranged in orthotropically arranged hyphal systems. Macroconidia, multi-celled, thick- and rough-walled, (sub)hyaline, clavate or cigar-shaped. Microconidia thin- and smooth-walled, hyaline, 1-celled, clavate. Sexual state produced after mating, arthroderma-like. Type species: *Microsporum cookei* Ajello.
1. ***Paraphyton cookei*** (Ajello) Gräser, Dukik & de Hoog, **comb. nov**.
Basionym: *Microsporum cookei* Ajello, Mycologia 51: 71, 1959. Type strain: CBS 228.58 = CDC B-276, from soil, Kentucky, USA, L. Ajello. Geophilic species.
Fac. syn.: *Nannizzia cajetani* Ajello, Sabouraudia 1: 175, 1961 ≡ *Arthroderma cajetani* (Ajello) Ajello, Weitzman, McGinnis & Padhye, in Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 513, 1986. Neotype: CDC B-4218; type strain: ATCC 14386 = CDC B-433 (crossing of ATCC 14387 (MT+) x ATCC 14388 (MT-)), from soil, Michigan, USA, L. Ajello.
Fac. syn.: *Microsporum racemosum* Borelli, Acta Méd. Venez. 12: 150, 1965 ≡ *Nannizzia racemosa* (Borelli) Rush-Munro, J.M.B. Smith & Borelli, Mycologia 62: 858, 1970 ≡ *Arthroderma racemosa* (Rush-Munro, J.M.B. Smith & Borelli) Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 514, 1986. Holotype: IMI 128984, crossing of CBS 424.74 = ATCC 18911 = CDC X-903 = IHEM 3452 = IMI 135823 = NCPF 484 = UAMH 3368 (MT-) 9 CBS 423.74 = ATCC 18910 = CDC X-902 = IHEM 3453 = IMI 135822 = NCPF 483 = UAMH 3367 (MT+), from soil, Georgia, USA, A.A. Padhye.
2. ***Paraphyton cookiellum*** (de Clerq) Gräser, Dukik & de Hoog, **comb. nov**.
Basionym: *Nannizzia cookiella* de Clercq, Mycotaxon 18: 24, 1983 ≡ *Arthroderma cookiellum* (de Clercq) Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 513, 1986. Holotype CBS-H 7397, type strains: CBS 101.83 (MT-) x CBS 102.83(MT+), from soil, Abidjan, Ivory Coast, D. de Clercq, 1984. Geophilic species.
3. ***Paraphyton mirabile*** (J.S. Choi, Gräser, Walther, Peano, Symoens & de Hoog) Gräser, Dukik & de Hoog, **comb. nov**.
Basionym: *Arthroderma mirabile* J.S. Choi, Gräser, Walther, Peano, Symoens & deHoog, Med. Mycol. 50: 168, 2012 ≡ *Microsporum mirabile* J.S. Choi, Gräser, Walther, Peano, Symoens & de Hoog, Med. Mycol. 50: 168, 2012. Holotype CBS H-20571, cross of CBS 124422 = IHEM 24407, from pelt of wild chamois, Italy, A. Peano (MT+) x CBS 129179 = IHEM 24409, from human toenail, The Netherlands (MT-). Zoophilic species (66).

Clade E: *Lophophyton*

Colonies expanding, granular or velvety, with brownish to red pigments. Macroconidial in loose clusters, large, up to 60 lm in length, thick- and rough-walled, multiseptate. Microconidia

present. Sexual state produced after mating, arthroderma-like.
Type species: *Epidermophyton gallinae* Mégnin.
1. ***Lophophyton gallinae*** (Mégnin) Matruchot & Dassonville; *Epidermophyton gallinae* Mégnin, C. R. Soc. Biol. 33: 404, 1881 ≡ *Lophophyton gallinae* (Mégnin) Matruchot & Dassonville, Revue Gén. Bot. 11: 429, 1899 ≡ *Achorion gallinae* (Mégnin) Sabouraud, Malad. Cuir Chev. 3: 553, 1910 ≡ *Sabouraudites gallinae* (Mégnin) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 327, 1923 ≡ *Closteroaleuriospora gallinae* (Mégnin) Grigoraki, Annls Sci. Nat., Bot., Sér. 10, 7: 412, 1925 ≡ *Microsporum gallinae* (Mégnin) Grigoraki, Annls Derm. Syph., Sér. 6, 10: 42, 1929 ≡ *Trichophyton gallinae* (Mégnin) Georg, Mycologia 44: 486, 1952. **Neotype designated herewith**: CBS 300.52, F. Blank. Zoophilic species on poultry.
Fac. syn.: *Nannizzia grubyi* Georg, Ajello, Friedman & Brinkman, Sabouraudia 1:194, 1962: *Arthroderma grubyi* (Georg, Ajello, Friedman & Brinkman) Ajello, Weitzman, McGinnis & Padhye, in Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 513, 1986. Neotype: CDC B-4219 (= CDC X-322 = CBS 243.66 = ATCC 14419 = IMI 113720 = NCPF 487, from dog ringworm, Missouri, USA, A.E.Blum x CDC X-321, from human ringworm, USA, L. Friedman. Reference strains: ATCC 14422 = CDC X-470 = CBS 100083 (MT+) 9 ATCC 14423 (MT-), single ascospore isolates from cross of CBS 243.66 9 CDC X-321.
Fac. syn.: *Microsporum vanbreuseghemii* Georg, Ajello, Friedman & S.A. Brinkman, Sabouraudia 1: 191, 1961/62. Type strain: CBS 243.66 = ATCC 14419 = CDC X-322 = IMI 113720 = NCPF 487, from dog ringworm, Missouri, USA, A.E. Blum.

Clade F: *Microsporum*

Colonies mostly granular to cottony, yellowish to brownish, with a cream-colored or brown colony reverse. Hyphae thin-walled, hyaline. Thallic macroconidia and microconidia, if present, arranged in orthotropically arranged hyphal systems. Macroconidia, multi-celled, thick- and rough-walled, (sub)hyaline, clavate, fusiform or cigar-shaped. Microconidia thin- and smooth-walled, hyaline, 1-celled, clavate. Sexual state sometimes produced after mating, arthroderma-like. Note that the three currently accepted species cannot be reliably distinguished by multilocus sequence analysis (Fig. 5); more detailed species are needed to establish species borderlines.
Type species: *Microsporum audouinii* Gruby.
1. ***Microsporum audouinii*** Gruby, C.R. Hebd. Séanc. Acad. Sci., Paris 17: 301, 1843 ≡ *Sporotrichum* audouinii (Gruby) Saccardo, Syll. Fung. 4: 101, 1886 ≡ *Sabouraudites audouinii* (Gruby) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 327, 1923 ≡ *Closteroaleurosporia audouinii* (Gruby) Grigoraki, Annls Sci. Nat., Bot., Sér. 10, 7: 412, 1925 ≡ *Veronaia audouinii* (Gruby) Benedek, Mycopath. Mycol. Appl. 14: 115, 1961. Neotype strain: CBS 545.93, from human skin, The Netherlands. Anthropophilic species.
Fac. syn.: *Sabouraudites langeronii* Vanbreuseghem, Annls Parasit. Hum. Comp. 25: 516, 1950. Authentic strain: CBS 404.61, from human, Zaire, R. Vanbreuseghem.
2. ***Microsporum canis*** (Bodin) Bodin; *Microsporum audouinii* Gruby var. *canis* Bodin, in Besnier, Brocq & Jacquet, Prat. Derm. p. 810, 1900 ≡ *Microsporum canis* (Bodin) Bodin, Champ. Paras. Homme p. 137, 1902 ≡ *Sabouraudites canis* (Bodin) Langeron, Précis Mycol. p. 534, 1945. Neotype strain: CBS 496.86, from feline ringworm, Japan, M. Hironaga. Zoophilic species.
Fac. syn.: *Nannizzia otae* Hasegawa & Usui, Jpn. J. Med. Mycol. 16: 151, 1975 ≡ *Arthroderma*

otae (Hasegawa & Usui) McGinnis, Weitzman, Padhye & Ajello, in Weitzman, McGinnis, Padhye & Ajello, Mycotaxon 25: 514, 1986. Holotype: VMUT-1, cross of monascospore cultures VUT-73015 = ATCC 28327 x VUT-74001 = ATCC 28328; reference strains CBS 495.86 = VUT-77054 (MT+) x CBS 496.86 = VUT 77055 (MT-), from feline ringworm, Japan, M. Hironaga. Note that the two mating partners are rather remote from each other, syntypes being located in *M. canis* and *M. audouinii* clusters (Fig. 5).
Fac. syn.: *Microsporum distortum* di Menna & Marples, Trans. Br. Mycol. Soc. 37: 372, 1954 ≡ *Microsporum canis* Bodin var. *distortum* (di Menna & Marples) Matsumoto, Padhye & Ajello, Trans. Br. Mycol. Soc. 81: 649, 1983. Type strain: CBS 101514 = NCPF 215, from human tinea capitis, New Zealand.

3. ***Microsporum ferrugineum*** Ota, Jpn. J. Derm. Urol. 21: 201, 1921 ≡ *Grubyella ferruginea* (Ota) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 330, 1923 ≡ *Arthrosporia ferruginea* (Ota) Grigoraki, Annls Sci. Nat., Bot., Sér. 10, 7: 414, 1925 ≡ *Achorion ferrugineum* (Ota) Guiart & Grigoraki, Lyon Méd. 141: 377, 1928 ≡ *Trichophyton ferrugineum* (Ota) Talice, Annls Parasit. Hum. Comp. 9: 83, 1931. Authentic strain: CBS 497.48, from human skin, Japan, M. Ota. Anthropophilic species.

Clade G: *Arthroderma*

Colonies mostly granular to cottony, yellowish to brownish, with a cream-colored or brown colony reverse. Hyphae thin-walled, hyaline. Thallic macroconidia and microconidia, if present, arranged in orthotropically arranged hyphal systems. Macroconidia, multi-celled, thick- and rough-walled, (sub)hyaline, clavate, fusiform or cigar-shaped. Microconidia thin- and smooth-walled, hyaline, 1-celled, clavate. Sexual state sometimes produced after mating, arthroderma-like.
Type species: *Arthroderma curreyi* Berkeley.

1. ***Arthroderma amazonicum*** (Moraes, Borelli & Feo) Gräser & de Hoog, **comb. nov**.
Basionym: *Microsporum amazonicum* Moraes, Borelli & Feo, Med. Cután. 11: 284, 1967. Type strain: CBS 967.68 = ATCC 18393, from hair of *Oryzomys* rat, Manaus, Brazil.
Fac.syn.: *Nannizzia borellii* Moraes, Padhye & Ajello, Mycologia 67: 1112, 1976 ≡ *Arthroderma borellii* (Moraes, Padhye & Ajello) Padhye, Weitzman, McGinnis & Ajello, in Weitzman, Mycotaxon 25: 513, 1986. Holotype CDC B-2093, cross of CDC B-2087 x B-2089, reference strains CBS 221.75 = ATCC 28356 = CDC Y-81 = IHEM 3454 (MT+) x ATCC 28357 = CDCY-82 = IHEM 3455 (MT-), all from fur of spiny rat (*Proechimys guannensis*), Belém, Brazil. The correct name of strain CBS 967.68 is *Arthroderma borellii*. Zoophilic species. The species is located in an ancestral position to the *Arthrodermataceae*; its taxonomy requires further study.

2. ***Arthroderma ciferrii*** Varsavsky & Ajello; *Trichophyton georgiae* Varsavsky & Ajello, Riv. Patol. Veg., Pavia, Sér. 3, 4: 357, 1964 ≡ *Arthroderma ciferrii* Varsavsky & Ajello, Riv. Patol. Veg., Pavia, Sér. 3, 4: 358, 1964 ≡ *Chrysosporium georgiae* (Varsavsky & Ajello) v. Oorschot, Stud. Mycol. 20: 31, 1980. Type strain: CBS 272.66 = UAMH 2534, from soil, Arkansas, USA, L. Ajello. Geophilic species.

3. ***Arthroderma cuniculi*** Dawson, Sabouraudia 2: 187, 1963. Holotype: IMI 96243, cross of single ascospore strains CBS 492.71 = ATCC 28442 = IHEM 4437 = IMI 96244 = NCPF 525A (MT-), from soil and hair of rabbit, Scotland, UK, C.O. Dawson x CBS 495.71 = ATCC 18444 = IMI 96245 (MT+), from soil and hair of rabbit, Scotland, UK, C.O. Dawson. Geophilic species.

4. ***Arthroderma curreyi*** Berkeley, Outl. Brit. Fung. p. 357, 1860. **Epitype, designated herewith**: CBS 353.66, from dune soil, UK, A.E. Apinis, 1966. Geophilic species.

5. ***Arthroderma eboreum*** (Brasch & Gräser) Gräser & de Hoog, **comb. nov**.

Basionym: *Trichophyton eboreum* Brasch & Gräser, J. Clin. Microbiol. 43: 5235, 2005. Type strain: CBS 117155 = DSM 16978, from human skin, Ivory Coast, J. Brasch.

Fac. syn.: *Arthroderma olidum* Cambell, Borman, Linton, Bridge & Johnson, Med. Mycol. 44: 457, 2006. Holotype: NCPF 5111, type strain NCPF 5088, crossing of NCPF 5102 x NCPF 5104, from badger hole soil, UK

6. ***Arthroderma flavescens*** R.G. Rees, Sabouraudia 5: 206, 1967 ≡ *Trichophyton flavescens* Padhye & Carmichael, Can. J. Bot. 49: 1535, 1971. Type strain: IMI 117342, crossing of IMI 112079, from feather of lorikeet (*Trichoglossus moluccanus*), Queensland, Australia, R.G. Rees, x IMI 117341 = CBS 473.78, from feather of sacred kingfisher (*Halycon sancta*), Queensland, Australia, R.G. Rees. The anamorph was later introduced for one of the strains producing the teleomorph. Zoophilic species.

7. ***Arthroderma gertleri*** Böhme, Mykosen 10: 251, 1967. Type: UAMH 2620, from soil, Germany, H. Böhme. Geophilic species.

Fac. syn.: *Trichophyton vanbreuseghemii* Rioux, Jarry & Juminez, Nat. Monspeliensia, Sér. Bot. 16: 158, 1964 (*non Arthroderma vanbreuseghemii* Takashio, Ann. Soc. Belg. Méd. Trop. 53: 547, 1973). Type strain: CBS 598.66, from soil, J.A. Rioux. The oldest name for this taxon is *T. vanbreuseghemii*, but the combination cannot be made because of an earlier homonym.

8. ***Arthroderma gloriae*** Ajello & Cheng; *Trichophyton gloriae* Ajello, in Ajello & Cheng, Mycologia 59: 257, 1967 ≡ *Arthroderma gloriae* Ajello & Cheng, Mycologia 59: 257, 1967. Type strain anamorph: CBS 228.79 = CDC X-138 = ATCC 16655, type strain teleomorph: crossing of CBS 664.77 = CDC X779 = UAMH 2820 = ATCC 16657 (MT+) x CBS 663.77 = CDC X780 = ATCC 16658 (MT-), from soil, Arizona, USA Geophilic species.

9. ***Arthroderma insingulare*** Padhye & Carmichael, Sabouraudia 10: 49, 1972. Reference strains: CBS 521.71 = ATCC 22519 = UAMH 3441 (MT A), from soil, Alberta, Canada, A.A. Padhye; CBS 522.71 = ATCC 22520 = IMI 158874 = NCPF 470 = UAMH 3442 (MT a), from soil, Alberta, Canada, A.A. Padhye.

10. ***Arthroderma lenticulare*** Pore, Tsao & Plunkett, Mycologia 57: 970, 1965. Reference strains: CBS 307.65 = ATCC 18445 = IHEM 3717 (MT+) x CBS 308.65 = ATCC 18446 = IHEM 3703 (MT-), both from soil of gopher hole, Los Angeles County, USA, R.S. Pore. Geophilic species.

11. ***Arthroderma melis*** Křivanec, Janečková & Otčenášek, Česká Mykol. 31: 92, 1977. Type strain: CBS 669.80, from burrow of badger (*Melis melis*), Moravia, Czech Republic. Geophilic species; no growth at 37 ºC.

12. ***Arthroderma multifidum*** Dawson, Sabouraudia 2: 189, 1963. Syntype strains: CBS 419.71 = ATCC 18440 = IHEM 4432 = IMI 094205 (MT+) x CBS 420.71 = ATCC 18441 = IMI 094206 (MT-), both from soil and hair from rabbit burrow, UK, C.O. Dawson. Geophilic species.

13. ***Arthroderma onychocola*** (Cmokova, Hubka, Skorepova & Kolařík) Gräser & de Hoog, **comb. nov.** Basionym: *Trichophyton onychocola* Cmokova, Hubka, Skorepova & Kolařík, Med. Mycol. 52: 287, 2014. Type strain: CBS 132920, from human nail, Czechia. Anthropophilic species (67).

14. ***Arthroderma phaseoliforme*** (Borelli & Feo) Gräser & de Hoog, **comb. nov**. Basionym: *Trichophyton phaseoliforme* Borelli & Feo, Acta Méd. Venez. 13: 176, 1966. Type strain: CBS

364.66, from pelt of mountain rat (*Proechimys guyanensis*), Venezuela. Geophilic species (68).

15. ***Arthroderma quadrifidum*** Dawson & Gentles, Sabouraudia 1: 35, 1961. Type not indicated; authentic strains CBS 117.61 (MT+) x CBS 118.61 (MT-), sent by C.O. Dawson & J.C. Gentles, 1961. Geophilic species.

16. ***Arthroderma redellii*** (Minnis, Lorch, D.L. Lindner & Blehert) Gräser & de Hoog, **comb. nov**.

Basionym: *Trichophyton redellii* Minnis, Lorch, D.L. Lindner & Blehert, in Lorch, Minnis, Meteyer, Redelli, White, Kaarakka, Muller, Lindner, Verant, Shearn-Bochsler & Blehert, J. Wildlife Dis. 51: 43, 2015. Type strain: CBS 134551 = CFMR 44738-03H, wing of hibernating bat (*Myotis lucifugus*), Wisconsin, USA, M.L. Verant, February 2012. Zoophilic species.

17. ***Arthroderma silverae*** Currah, S.P. Abbott & Sigler, Mycol. Res. 100: 195, 1996. Type: UAHM 6517 (69). The strain was not available for study.

18. ***Arthroderma thuringiensis*** (Koch) Gräser & de Hoog, **comb. nov**. Basionym: *Trichophyton thuringiense* Koch, Mykosen 12: 288, 1969. Type strain: CBS 417.71 = ATCC 22648 = IMI 134993 = NCPF 492A = UAMH, from mouse skin, Germany, H.A. Koch, 1964. Zoo- or geophilic species (7).

19. ***Arthroderma tuberculatum*** Kuehn, Mycopath. Mycol. Appl. 13: 190, 1960. Type strain: CBS 473.77 = ATCC 26700 = UAMH 873, feather of *Turdus americanus*, Illinois, USA, H.H. Kuhn. Geophilic species.

20. ***Arthroderma uncinatum*** Dawson & Gentles, Sabouraudia 1: 55, 1961. Syntypes: CBS 315.65 (MT+), CBS 316.65 (MT-), both from soil, California, USA, O.A. Plunkett. Geophilic species.

Fac. syn.: *Keratinomyces ajelloi* Vanbreuseghem, Bull. Acad. R. Méd. Belg. 38: 1075, 1952 ≡ *Epidermophyton terrigenum* Evolceanu & Alteras, Mycopath. Mycol. Appl. 11: 202, 1959 (name change) ≡ *Microsporum ajelloi* (Vanbreuseghem) Arievitch & Stiepanishchewa, Proc. Int. Symp. Med. Mycol.,Warsaw p. 43, 1965 ≡ *Trichophyton ajelloi* (Vanbreuseghem) Ajello, Sabouraudia 6: 148, 1966 ≡ *Epidermophyton ajelloi* (Vanbreuseghem) Novák & Galgóczy, Acta Bot. Hung.15:130, 1969. Type strain: CBS101515 = NCPF 216, from soil, Belgium, R. Vanbreuseghem. Fac. syn.: *Epidermophyton stockdaleae* Prochacki & Engelhardt-Zasada, Mycopathologia 54: 342, 1974. Type strain: CBS 128.75, from soil, Poland, C. Engelhardt.

Fac. syn.: *Keratinomyces ajelloi* Vanbreuseghem var. *nanum* Kunert & Hejtmánek, Česká Epid. Mikrobiol. Immunol. 13: 296, 1964 ≡ *Trichophyton ajelloi* (Vanbreuseghem) Ajello var. *nanum* (Kunert & Hejtmánek) Ajello, Sabouraudia 6: 148, 1966. Type strain: CBS 180.64 = ATCC 22398 = NCPF 473, from soil, Czechoslovakia, M. Hejtmánek, 1964.

21. ***Arthroderma vespertilii*** (Guarro, Vidal & De Vroey) Gräser & de Hoog, **comb. nov**.

Basionym: *Chrysosporium vespertilii* Guarro, Vidal & De Vroey, in Vidal, Guarro & De Vroey, Mycotaxon 59: 190, 1996. Type strain: CBS 355.93 = IMI 357403 = FMR 3752, from intestinal content of bat, Kibisi, near Kinshasa, Zaire (70). Zoophilic species.

List of doubtful dermatophyte names not listed as such in Atlas of Clinical Fungi

castellanii—*Veronaia castellanii* Benedek, Mycopath. Mycol. Appl. 14: 115, 1961. Type material not known to be preserved; identity doubtful.

ceretanicus—*Keratinomyces ceretanicus* Punsola & Guarro, Mycopathologia 85: 185, 1984. Type strain: CBS 269.89 = FMR 3063, from soil, Valdivia, Chile, J. Guarro, Nov. 1988. The type species of Keratinomyces, *K. ajelloi* clusters in *Arthroderma*. *Keratinomyces ceretanicus*

is a phylogenetically distant, psychrophilic soil fungus in the *Onygenace*. We propose the following, as yet monotypic genus for this fungus:

Guarromyces Gräser & de Hoog, **gen. nov**.

Macroconidia hyaline, smooth- and thick-walled, lanceolate to cylindrical, multiseptate, borne holothallically in loose clusters on creeping hyphae; microconidia absent. Type species: ***Guarromyces ceretanicus*** (Punsola & Guarro) Gräser & de Hoog, **comb. nov**.

granulosum—*Trichophyton granulosum* Sabouraud, in Pécus, Rev. Gén. Méd. Vét. 15: 561, 1909: *Trichophyton mentagrophytes* (Robin) Blanchard var. *granulosum* (Sabouraud) Neveu-Lemaire, Précis Parasitol. Anim. Domest. p. 71, 1912: *Ectotrichophyton granulosum* (Sabouraud) Castellani & Chalmers, Man. Trop. Med., ed. 3, p. 1006, 1919: *Sabouraudites granulosus* (Sabouraud) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 328, 1923: *Chlamydoaleuriospora granulosa* (Sabouraud) Grigoraki, Annls Sci. Nat., Bot., Sér. 10, 7: 412, 1925: *Trichophyton gypseum* Bodin var. *granulosum* (Sabouraud) Frágner, Česká Mykol.10: 108, 1956. In general this species is treated as a heavily sporulating variant of *T. mentagrophytes* occurring on cats and dogs (52). As no type material is known to be preserved, its identity remains doubtful.

gypseum—*Trichophyton gypseum* Bodin, Champ. Paras. Homme p. 115, 1902: *Achorion gypseum* (Bodin) Bodin, Annls Derm. Syph. 4: 585, 1907: *Sabouraudites gypseus* (Bodin) Ota & Langeron, Annls Parasit. Hum. Comp. 1: 328, 1923: *Closterosporia gypsea* (Bodin) Grigoraki, Annls Sci. Nat., Bot., Sér. 10, 7: 411, 1925: *Microsporum gypseum* (Bodin) Guiart & Grigoraki, Lyon Méd. 141: 377, 1928: *Trichophyton mentagrophytes* (Robin) Blanchard var. *gypseum* (Bodin) Kamyszek, Med. Weteryn. 24: 146, 1945. Type material not known to be preserved; doubtful species.

lanosa—*Closterosporia lanosa* Grigoraki, C. R. Hebd. Séanc. Acad. Sci., Paris 179: 1424, 1924. Type material not known to be preserved; doubtful species.

microsporum—*Oidium microsporium* Kambayashi, Jpn. J. Derm. Urol. 21: 460, 1921. Type material not known to be preserved; identity doubtful.

serratus—*Ctenomyces serratus* Eidam, Eitr. Biol. Pfl. 3: 274, 1880. *Ctenomyces* is a gymnothecial genus of terrestrial fungi with chrysosporium-like conidia and has uncertain phylogenetic position. Several species have been classified in the genus. For a description, see Böhme (71).

terrestre—*Trichophyton terrestre* Durie & Frey, Mycologia 49: 401,1957. Type UAMH was not available for study. In literature the species has been listed as the anamorph of different *Arthroderma* species which on molecular grounds appear to be remote from each other. *Trichophyton terrestre* needs to be reevaluated.

terrestre-primum—*Trichophyton terrestre*-primum Szathmáry, Magya Orvosi Arch. 37-6: 1–6, 1936. Type material not known to be preserved; identity doubtful.

Epilogue

The present paper provides an evaluated list of currently accepted species in *Arthrodermataceae*, but is by no means exhaustive. Many groups require more detailed polyphasic studies with mating experiments to determine exact borderlines between species. Some extant types could not be acquired during the course of this study. New, genomic and proteomic studies will provide understanding of the observed clinical differences in predilection between closely related species. It is expected that among the geo- and zoophilicgroups numerous species are yet to be discovered in undersampled habitats; our review means to provide a new starting point for these subsequent studies.

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ST1.

	CBS number	Current taxon name	New taxon name	Status	Source
1	CBS 221.75	A. borellii	A. borellii		Rat
2	CBS 967.68	A. borellii	A. borellii	ST Nannizzia borellii	Bat
3	CBS 272.66	C. georgiae	A. ciferrii	T Arthroderma ciferrii	Soil
4	CBS 492.71	A. cuniculi	A. cuniculi	ST Arthroderma cuniculi	Rabbit burrow
5	CBS 495.71	A. cuniculi	A. cuniculi	ST Arthroderma cuniculi	Rabbit burrow
6	CBS 353.66	A. curreyi	A. curreyi	ET Arthroderma curreyi	Soil
7	CBS 117155	T. eboreum	A. eboreum	T Trichophyton eboreum	Skin
8	CBS 292.93	T. sp.	A. eboreum		Skin
9	CBS 473.78	A. flavescens	A. flavescens	ST Arthroderma flavescens	Kingfisher
10	CBS 474.78	A. flavescens	A. flavescens		
11	CBS 598.66	A. gertleri	A. gertleri	ST Trichophyton vanbreuseghemii	Soil
12	CBS 666.77	A. gertleri	A. gertleri		
13	CBS 228.79	A. gloriae	A. gloriae	T Arthroderma gloriae	Soil
14	CBS 663.77	A. gloriae	A. gloriae		
15	CBS 664.77	A. gloriae	A. gloriae		
16	CBS 521.71	A. insingulare	A. insingulare		Soil
17	CBS 522.71	A. insingulare	A. insingulare		Soil
18	CBS 307.65	A. lenticulare	A. lenticulare	T Arthroderma lenticulare	Gopher burrow
19	CBS 308.65	A. lenticulare	A. lenticulare	T Arthroderma lenticulare	Gopher burrow
20	CBS 120.30	T. tonsurans	A. melis		Human
21	CBS 669.80	A. melis	A. melis	T Arthroderma melis	Badger burrow
22	CBS 419.71	A. multifidum	A. multifidum	ST Arthroderma multifidum	Rabbit burrow
23	CBS 420.71	A. multifidum	A. multifidum	ST Arthroderma multifidum	Rabbit burrow
24	CBS 132920	T. sp.	A. onychocola	T Trichophyton onychocola	Human
25	CBS 364.66	T. phaseoliforme	A. phaseoliforme	ST Trichophyton phaseoliforme	Mountain rat
26	CBS 117.61	A. quadrifidum	A. quadrifidum	AUT Arthroderma quadrifidum	Soil
27	CBS 118.61	A. quadrifidum	A. quadrifidum	AUT Arthroderma quadrifidum	Soil
28	CBS 138.26	A. curreyi	A. quadrifidum		
29	CBS 310.65	A. quadrifidum	A. quadrifidum		Soil
30	CBS 311.65	A. quadrifidum	A. quadrifidum		Soil
31	CBS 134551	T. redellii	A. redellii	T Trichophyton redellii	Bat
32	CBS 132929	T. thuringiense	A. sp.		Nail
33	CBS 417.71	T. thuringiense	A. thuringiense	T Trichophyton thuringiense	Mouse
34	CBS 473.77	A. tuberculatum	A. tuberculatum	T Arthroderma tuberculatum	Blackbird
35	CBS 101515	A. uncinatum	A. uncinatum	T Keratinomyces ajelloi	Soil
36	CBS 119779	T. ajelloi var. ajelloi	A. uncinatum		Nail
37	CBS 128.75	A. uncinatum	A. uncinatum	ST E. stockdaleae	Soil
38	CBS 179.57	A. uncinatum	A. uncinatum		Soil
39	CBS 180.57	A. uncinatum	A. uncinatum		Soil
40	CBS 180.64	A. uncinatum	A. uncinatum	ST Keratinomyces ajelloi var. nanum	Soil
41	CBS 315.65	A. uncinatum	A. uncinatum	ST Arthroderma uncinatum	Soil
42	CBS 316.65	A. uncinatum	A. uncinatum	ST Arthroderma uncinatum	Soil
43	CBS 355.93	C. vespertilii	A. vespertilii	T Chrysosporium vespertilium	Bat intestine
44	CBS 187.61	Ctenomyces serratus	Ctenomyces serratus	NT Ctenomyces serratus	Soil
45	CBS 544.63	Ctenomyces serratus	Ctenomyces serratus		Soil

ST1. (Continued)

	CBS number	Current taxon name	New taxon name	Status	Source
46	CBS 100148	A. uncinatum	E. floccosum		Skin
47	CBS 108.67	E. floccosum var. floccosum	E. floccosum		Human
48	CBS 230.76	E. floccosum var. floccosum	E. floccosum	NT Epidermophyton floccosum	Human
49	CBS 240.67	E. floccosum var. floccosum	E. floccosum		Skin
50	CBS 457.65	E. floccosum var. nigricans	E. floccosum		
51	CBS 553.84	E. floccosum var. floccosum	E. floccosum		Human
52	CBS 269.89	Keratinomyces ceretanicus	Guarromyces ceretanicus		
53	CBS 100083	A. grubyi	L. gallinae		
54	CBS 243.66	A. grubyi	L. gallinae	T Nannizzia grubyi	Dog
55	CBS 244.66	A. grubyi	L. gallinae		Scalp
56	CBS 300.52	A. grubyi	L. gallinae	NT Lophopyton gallinae	
57	CBS 545.93	M. audouinii	M. audouinii	NT Microsporum audouinii	Scalp
58	CBS 495.86	A. otae	M. audouinii	T Nannizzia otae	
59	CBS 102894	M. audouinii	M. audouinii		Scalp
60	CBS 108932	M. audouinii	M. audouinii		
61	CBS 108933	M. audouinii	M. audouinii		Human
62	CBS 108934	M. audouinii	M. audouinii		Human
63	CBS 119449	M. audouinii	M. audouinii		Scalp
64	CBS 404.61	M. audouinii	M. audouinii	AUT Sabouraudites langeronii	Human
65	CBS 101514	A. otae	M. canis	T Microsporum distortum	Scalp
66	CBS 114329	M. canis	M. canis		Skin
67	CBS 130922	M. canis	M. canis		Skin
68	CBS 130931	M. canis	M. canis		Skin
69	CBS 130932	M. canis	M. canis		Skin
70	CBS 130949	M. canis	M. canis		Human
71	CBS 156.69	A. otae	M. canis		Skin
72	CBS 191.57	A. otae	M. canis		Dog
73	CBS 214.79	A. otae	M. canis		Rabbit
74	CBS 217.69	A. otae	M. canis		Nail
75	CBS 238.67	A. otae	M. canis		Human
76	CBS 274.62	A. otae	M. canis		Monkey
77	CBS 281.63	A. otae	M. canis		Scalp
78	CBS 283.63	A. otae	M. canis		
79	CBS 284.63	A. otae	M. canis		Gibbon
80	CBS 445.51	M. ferrugineum	M. canis		
81	CBS 482.76	A. otae	M. canis		Skin
82	CBS 496.86	A. otae	M. canis	ST Nannizzia otae, NT Microsporum canis	Cat
83	CBS 109478	M. audouinii	M. canis		Scalp
84	CBS 317.31	M. ferrugineum	M. ferrugineum		
85	CBS 373.71	M. ferrugineum	M. ferrugineum		Human
86	CBS 449.61	M. ferrugineum	M. ferrugineum		
87	CBS 452.59	T. concentricum	M. ferrugineum		Skin
88	CBS 497.48	M. ferrugineum	M. ferrugineum		Scalp

ST1. (Continued)

	CBS number	Current taxon name	New taxon name	Status	Source
89	CBS 366.81	A. corniculatum	N. corniculata	ST Nannizzia corniculata	Soil
90	CBS 364.81	A. corniculatum	N. corniculata	ST Nannizzia corniculata	Soil
91	CBS 349.49	M. duboisii	N. duboisii	T Sabouraudites duboisii	Skin
92	CBS 599.66	A. fulvum	N. fulva	T Microsporum boullardii	Soil
93	CBS 130934	M. fulvum	N. fulva		Soil
94	CBS 130942	M. fulvum	N. fulva		Human
95	CBS 146.66	M. gypseum	N. fulva	AUT Favomicrosporon pinettii	Contaminant
96	CBS 147.66	M. gypseum	N. fulva	AUT Favomicrosporon pinettii	Contaminant
97	CBS 243.64	A. fulvum	N. fulva	T Keratinomyces longifusus	Scalp
98	CBS 287.55	A. fulvum	N. fulva	T Microsporum fulvum	Human
99	CBS 385.64	A. otae	N. fulva		Human
100	CBS 529.71	A. fulvum	N. fulva	T Microsporum ripariae	Birdnest
101	CBS 120675	M. gypseum	N. gypsea		
102	CBS 100.64	M. gypseum var. vinosum	N. gypsea	ST Microsporum gypseum var. vinosum	Skin
103	CBS 118893	M. gypseum	N. gypsea		Skin
104	CBS 130936	M. gypseum	N. gypsea		Skin
105	CBS 130939	M. gypseum	N. gypsea		Skin
106	CBS 171.64	A. gypseum	N. gypsea		Soil
107	CBS 258.61	A. gypseum	N. gypsea	NT Gymnoascus gypseus	Soil
108	CBS 130948	M. gypseum	N. incurvata		Skin
109	CBS 173.64	A. incurvatum	N. incurvata		Skin
110	CBS 174.64	A. incurvatum	N. incurvata	T Nannizzia incurvata	Skin
111	CBS 314.54	A. obtusum	N. nana	T Microsporum gypseum var. nanum	Scalp
112	CBS 321.61	A. obtusum	N. nana	ST Nannizzia obtusa	Human
113	CBS 322.61	A. obtusum	N. nana	ST Nannizzia obtusa	Human
114	CBS 632.82	A. obtusum	N. nana		Human
115	CBS 421.74	A. persicolor	N. persicolor		
116	CBS 871.70	A. persicolor	N. persicolor	ST Nannizzia quinckeana	Skin
117	CBS 288.55	M. praecox	N. praecox	AUT Microsporum praecox	Human
118	CBS 128066	M. praecox	N. praecox		Skin, from horse
119	CBS 128067	M. praecox	N. praecox		Skin, from horse
120	CBS 121947	M. amazonicum	N. species		Skin
121	CBS 450.65	A. racemosum	N. species	T Microsporum racemosum	Rat
122	CBS 130935	M. racemosum	P. cookei		Soil
123	CBS 227.58	A. cajetanum	P. cookei		
124	CBS 228.58	A. cajetanum	P. cookei	T Microsporum cookei	Soil
125	CBS 337.74	A. cajetanum	P. cookei		Soil
126	CBS 423.74	A. racemosum	P. cookei	ST Nannizzia racemosa	Soil
127	CBS 424.74	A. racemosum	P. cookei	ST Nannizzia racemosa	Soil
128	CBS 101.83	A. cookiellum	P. cookiellum	ST Nannizzia cookiella	Soil
129	CBS 102.83	A. cookiellum	P. cookiellum	ST Nannizzia cookiella	Soil
130	CBS 124422	M. mirabile	P. mirabile	ST Microsporum mirabile	Chamois
131	CBS 129179	M. mirabile	P. mirabile	ST Microsporum mirabile	Nail
132	CBS 646.73	A. vanbreuseghemii	T. vanbreuseghemii	T Arthroderma vanbreuseghemii	
133	CBS 809.72	A. benhamiae	T. benhamiae		
134	CBS 112368	A. benhamiae	T. benhamiae		Skin, from guinea pig
135	CBS 112369	A. benhamiae	T. benhamiae		Skin, from guinea pig
136	CBS 112370	A. benhamiae	T. benhamiae		Skin, from guinea pig

ST1. (Continued)

	CBS number	Current taxon name	New taxon name	Status	Source
137	CBS 112371	A. benhamiae	T. benhamiae		Skin, from guinea pig
138	CBS 112857	A. benhamiae	T. benhamiae		Skin, from guinea pig
139	CBS 112859	A. benhamiae	T. benhamiae		Skin, from rabbit
140	CBS 120669	T. mentagrophytes	T. benhamiae		Guinea pig
141	CBS 280.83	A. benhamiae	T. benhamiae		Skin
142	CBS 623.66	A. benhamiae	T. benhamiae	ST Arthroderma benhamiae	Human
143	CBS 624.66	A. benhamiae	T. benhamiae	ST Arthroderma benhamiae	
144	CBS 806.72	A. benhamiae	T. benhamiae		Guinea pig
145	CBS 934.73	A. benhamiae	T. benhamiae		
146	CBS 131645	T. bullosum	T. bullosum		Skin
147	CBS 363.35	T. bullosum	T. bullosum	T Trichophyton bullosum	Horse
148	CBS 557.50	T. bullosum	T. bullosum		
149	CBS 196.26	T. concentricum	T. concentricum	NT Trichophyton concentricum	Skin
150	CBS 448.61	T. concentricum	T. concentricum		Skin
151	CBS 563.83	T. concentricum	T. concentricum		Skin
152	CBS 109036	T. equinum	T. equinum		Skin
153	CBS 100080	T. equinum	T. equinum	T Trichophyton equinum var. autotrophicum	Horse
154	CBS 270.66	T. equinum	T. equinum	NT Trichophyton equinum	Horse
155	CBS 285.30	T. tonsurans	T. equinum	T Trichophyton areolatum	
156	CBS 634.82	T. equinum	T. equinum		Horse
157	CBS 344.79	T. erinacei	T. erinacei		Skin
158	CBS 474.76	T. erinacei	T. erinacei	T Trichophyton proliferans	Skin
159	CBS 511.73	T. erinacei	T. erinacei	T Trichophyton mentagrophytes var. erinacei	Hedghog
160	CBS 124411	T. sp.	T. erinacei		Dog
161	CBS 220.25	T. eriotrephon	T. eriotrephon	T Trichophyton eriotrephon	Skin
162	CBS 108.91	T. erinacei	T. interdigitale		
163	CBS 110.65	T. mentagrophytes	T. interdigitale		Groin
164	CBS 113880	T. mentagrophytes	T. interdigitale		Nail
165	CBS 117723	A. vanbreuseghemii	T. interdigitale		Skin
166	CBS 119447	T. violaceum	T. interdigitale		Scalp
167	CBS 232.76	A. vanbreuseghemii	T. interdigitale		Skin
168	CBS 425.63	A. vanbreuseghemii	T. interdigitale	T Trichophyton batonrougei	
169	CBS 428.63	A. vanbreuseghemii	T. interdigitale	NT Trichophyton interdigitale	Skin
170	CBS 449.74	A. vanbreuseghemii	T. interdigitale		Skin
171	CBS 475.93	A. vanbreuseghemii	T. interdigitale	T Trichophyton krajdenii	Skin
172	CBS 559.66	A. vanbreuseghemii	T. interdigitale		Skin
173	CBS 647.73	A. vanbreuseghemii	T. interdigitale	T Trichophyton candelabrum	Nail
174	CBS 124426	T. interdigitale	T. mentagrophytes		Dog
175	CBS 124410	T. interdigitale	T. mentagrophytes		Dog
176	CBS 124419	T. interdigitale	T. mentagrophytes		
177	CBS 124424	T. interdigitale	T. mentagrophytes		Chamois
178	CBS 124425	T. interdigitale	T. mentagrophytes		Cat
179	CBS 304.38	T. radicosum	T. mentagrophytes	T Trichophyton radicosum	
180	IHEM 4268	T. mentagrophytes	T. mentagrophytes	NT Trichophyton mentagrophytes	
181	CBS 126.34	T. interdigitale	T. mentagrophytes	T Bodinia abyssinica	Skin
182	CBS 120324	T. mentagrophytes	T. mentagrophytes		Skin
183	CBS 120356	T. mentagrophytes	T. mentagrophytes		Scalp

ST1. (Continued)

	CBS number	Current taxon name	New taxon name	Status	Source
184	CBS 124401	A. benhamiae	T. mentagrophytes		Guinea pig
185	CBS 124404	T. interdigitale	T. mentagrophytes		Rabbit
186	CBS 124408	T. interdigitale	T. mentagrophytes		Dog
187	CBS 124415	T. interdigitale	T. mentagrophytes		Cat
188	CBS 124421	T. interdigitale	T. mentagrophytes		Rabbit
189	CBS 124420	T. interdigitale	T. mentagrophytes		Rabbit
190	CBS 120357	T. mentagrophytes	T. mentagrophytes		Scalp
191	CBS 158.66	T. mentagrophytes	T. quinckeanum		Skin
192	CBS 318.56	T. mentagrophytes	T. quinckeanum	NOT NT Microsporum mentagrophytes	Skin
193	IHEM 13697	T. quinckeanum	T. quinckeanum	NT Trichophyton quickeanum	Mouse
194	CBS 100081	T. rubrum	T. rubrum	T Trichophyton fischeri	Contaminant
195	CBS 100084	T. rubrum	T. rubrum	T Trichophyton raubitschekii	Skin
196	CBS 100238	T. rubrum	T. rubrum		
197	CBS 102856	T. rubrum	T. rubrum		Nail
198	CBS 110399	T. rubrum	T. rubrum		Skin
199	CBS 115314	T. rubrum	T. rubrum		Nail
200	CBS 115315	T. rubrum	T. rubrum		Skin
201	CBS 115316	T. rubrum	T. rubrum		Skin
202	CBS 115317	T. rubrum	T. rubrum		Human
203	CBS 115318	T. rubrum	T. rubrum		Nail
204	CBS 117539	T. rubrum var. flavum	T. rubrum		Nail
205	CBS 118892	T. rubrum	T. rubrum		Nail
206	CBS 289.86	T. rubrum	T. rubrum	T Trichophyton kanei	Skin
207	CBS 376.49	T. rubrum	T. rubrum	T Trichophyton rodhainii	Skin
208	CBS 392.58	T. rubrum	T. rubrum	NT Epidermophyton rubrum	Skin
209	CBS 592.68	T. rubrum	T. rubrum	T Trichophyton fluviomuniense	Skin
210	CBS 120425	T. rubrum	T. rubrum		Nail
211	CBS 202.88	T. rubrum	T. rubrum		Skin
212	CBS 288.86	T. rubrum	T. rubrum		Contaminant
213	CBS 118537	T. schoenleinii	T. schoenleinii		Scalp
214	CBS 118538	T. schoenleinii	T. schoenleinii		Scalp
215	CBS 118539	T. schoenleinii	T. schoenleinii		Scalp
216	CBS 433.63	T. schoenleinii	T. schoenleinii		Scalp
217	CBS 458.59	T. schoenleinii	T. schoenleinii	NT Trichophyton schoenleinii	Human
218	CBS 417.65	A. simii	T. simii		Poultry
219	CBS 448.65	A. simii	T. simii	ST Arthroderma simii	Poultry
220	CBS 449.65	A. simii	T. simii	ST Arthroderma simii	Poultry
221	CBS 520.75	A. simii	T. simii		Macaca
222	CBS 109033	T. tonsurans	T. tonsurans		Horse
223	CBS 109034	T. tonsurans	T. tonsurans		Horse
224	CBS 112186	T. tonsurans	T. tonsurans		Human
225	CBS 112187	T. tonsurans	T. tonsurans		Human
226	CBS 112188	T. equinum	T. tonsurans		Horse
227	CBS 112189	T. tonsurans	T. tonsurans		Human
228	CBS 112190	T. tonsurans	T. tonsurans		Horse
229	CBS 112191	T. tonsurans	T. tonsurans		Human
230	CBS 112192	T. tonsurans	T. tonsurans		Horse

ST1. (Continued)

	CBS number	Current taxon name	New taxon name	Status	Source
231	CBS 112193	T. equinum	T. tonsurans		Horse
232	CBS 112194	T. tonsurans	T. tonsurans		Horse
233	CBS 112195	T. tonsurans	T. tonsurans		Horse
234	CBS 112198	T. equinum	T. tonsurans		Human
235	CBS 112856	A. benhamiae	T. tonsurans		Scalp, zoo transmission
236	CBS 182.76	T. tonsurans	T. tonsurans		Horse
237	CBS 318.31	T. tonsurans	T. tonsurans	T Trichophyton floriforme	
238	CBS 338.37	T. immergens	T. tonsurans	T Trichophyton immergens	Skin
239	CBS 496.48	T. tonsurans	T. tonsurans	NT Trichophyton tonsurans	Scalp
240	CBS 130944	T. verrucosum	T. verrucosum		Scalp
241	CBS 130945	T. verrucosum	T. verrucosum		Skin
242	CBS 130946	T. verrucosum	T. verrucosum		Scalp
243	CBS 130947	T. verrucosum	T. verrucosum		Skin
244	CBS 134.66	T. verrucosum	T. verrucosum		Scalp
245	CBS 161.66	T. verrucosum	T. verrucosum		Skin
246	CBS 282.82	T. verrucosum	T. verrucosum		Cow
247	CBS 326.82	T. verrucosum	T. verrucosum		Cow
248	CBS 365.53	T. verrucosum	T. verrucosum	NT Trichophyton verrucosum	Cow
249	CBS 517.63	T. rubrum	T. violaceum	T Trichophyton kuryangei	Scalp
250	CBS 452.61	T. violaceum	T. violaceum		Scalp
251	CBS 118535	T. violaceum	T. violaceum		Scalp
252	CBS 119446	T. violaceum	T. violaceum		Scalp
253	CBS 120316	T. violaceum	T. violaceum		Scalp
254	CBS 120317	T. violaceum	T. violaceum		Scalp
255	CBS 120318	T. violaceum	T. violaceum		Scalp
256	CBS 120319	T. violaceum	T. violaceum		Scalp
257	CBS 120320	T. violaceum	T. violaceum		Scalp
258	CBS 178.91	T. sp.	T. violaceum		Nail
259	CBS 201.88	T. violaceum	T. violaceum		Skin
260	CBS 118548	M. ferrugineum	T. violaceum		Scalp
261	CBS 305.60	T. violaceum	T. violaceum	T Trichophyton yaoundei	Scalp
262	CBS 359.62	T. balcaneum	T. violaceum	T Trichophyton balcaneum	Human
263	CBS 374.92	T. violaceum	T. violaceum	NT Trichophyton violaceum	Skin
264	IHEM 19751	T. soudanense	T. violaceum	NT Trichophyton soudanense	Scalp

A = Arthroderma; AUT = authentic; C = Chrysosporium; ET = epitype; E = Epidermophyton; NT = neotype; M = Microsporum; ST = syntype; N = Nannizzia; T = (ex-)holotype; L = Lophophyton; P = Paraphyton

CHAPTER 3

Ultra-high resolution mass spectrometry of closely related dermatophytes with different clinical predilections

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Abstract

In the present study, an innovative top-down liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the identification of clinically relevant fungi is tested using a model set of dermatophyte strains. The methodology characterizes intact proteins derived from *Trichophyton* species which are used as parameters of differentiation. To test its resolving power compared to that of traditional Sanger sequencing and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF), twenty-four strains of closely related dermatophytes, *Trichophyton rubrum, T. violaceum*, *T. tonsurans, T. equinum* and *T. interdigitale*, were subjected to this new approach. Using tandem mass spectrometry (MS/MS) and different deconvolution algorithms, we identified hundreds of individual proteins, with a sub population of these used as strain- or species-specific markers. Three species, i.e. *T. rubrum*, *T. violaceum* and *T. interdigitale* were identified correctly down to the species level. Moreover, all isolates associated with these three species were identified correctly down to the strain level. In the *T. tonsurans-equinum* complex, eight out of twelve strains showed nearly identical proteomes, indicating an unresolved taxonomic conflict already apparent from previous phylogenetic data. In this case, it was determined with high probability that only a single species may be present. Our study successfully demonstrates applicability of the mass spectrometric approach to identify clinically relevant filamentous fungi. Herewith we present the first proof-of-principle study employing the mentioned technology to differentiate microbial pathogens. The ability to differentiate fungi at the strain level sets the stage to improve patient outcomes, such as for example, early detection of strains that carry resistance to antifungals.

Keywords

Mass spectrometry, dermatophytes

Introduction

Dermatophytes are fungi that are able to invade keratinized tissues, causing infections of the skin, hair and nails (1). Almost every human contracts at least one such infection in their lifetime. Due to this high incidence, over 500 million US dollars are spent annually on antimycotic treatment against dermatophytes (2). The prevalent species encountered in dermatology are classified in three genera: *Trichophyton*, *Microsporum* and *Epidermophyton*. *Trichophyton* in a modern sense comprises the anthropophilic species, along with species infecting domesticated animals (3).

In the routine clinical laboratory, the presentation of clinical symptoms, colony morphology, microscopic features, physiology or alternatively nucleic acid sequencing of the internal transcribed spacer (ITS) domain are commonly applied to dermatophyte identification. Following its successful adoption by many clinical laboratories, MALDI-TOF mass spectrometry has been applied to a broad range of species (4-9) or species groups of dermatophytes (10, 11). Even with these recent advances in identification and characterization using MALDI-TOF, accuracy rates remain at the 50 to 60 percent range with very high no-call rates (12), which is partly due to inadequate taxonomy of dermatophytes at the DNA level (3). In other fungal groups, identification of clinically relevant filamentous fungi has been shown to be possible with the addition of custom acquired data supplementing IVD databases (6, 7).

As an alternative strategy to the MALDI-TOF fingerprint approach, proteome-based strategies involve identification of proteins derived from microbial extracts. Two fundamentally different mass spectrometric strategies are available for protein identification: bottom-up and top-down. In bottom-up proteomics purified proteins or complex protein mixtures are subjected to proteolytic cleavage prior to MS-analysis. In top-down proteomics, intact protein ions or large protein fragments are injected directly into the mass spectrometer where they are further fragmented. The main advantage of top-down analysis is the ability to reveal intact protein masses, structural amino acid sequence variants and (combinations of) post-translational modifications.

In the present study, we utilize LC-MS/MS to separate proteins from dermatophyte extracts and analyze them sequentially in an Orbitrap tandem mass spectrometer. Amino acid sequence information obtained from tandem mass spectrometry is used to identify the observed proteins, which in turn leads to the correct classification of clinically relevant dermatophytes. This tandem mass spectrometric (MS/MS) process, termed collision-induced dissociation (CID), imparts excess energy to the intact protein ions, resulting in smaller mass amino acid sequence specific protein fragments which are used to directly identify any given protein undergoing this process. Several thousands of highly informative MS/MS spectra from the fragmented proteins or peptides are obtainable this way in a single run. The key difference of this approach when compared to fingerprinting/pattern recognition by MALDI-TOF, is the accurate assignment of intact protein and fragment masses that allows for statistically relevant high confidence protein identification (13). These identified proteins in turn, either singly or in combination, can be used as diagnostic markers of clinically relevant microorganisms.

The goal of the present study was to provide a proof-of-principle experiment employing Orbitrap LC-MS/MS for discrimination of filamentous fungi and to establish a proteomic approach for detailed characterization of strain diversity of the investigated taxa. As a model, two closely related but different pairs of species were compared. The members of one species pair are known to belong to unambiguously different species, whereas the separation of the other pair or lineages is doubtful, possibly comprising only a single species (Fig. 1). The former set concerns the *Trichophyton rubrum* group, comprising two species: *T. rubrum*, with a global prevalence and mainly causing tinea corporis and tinea pedis, and *T. violaceum*, which mostly causes tinea capitis

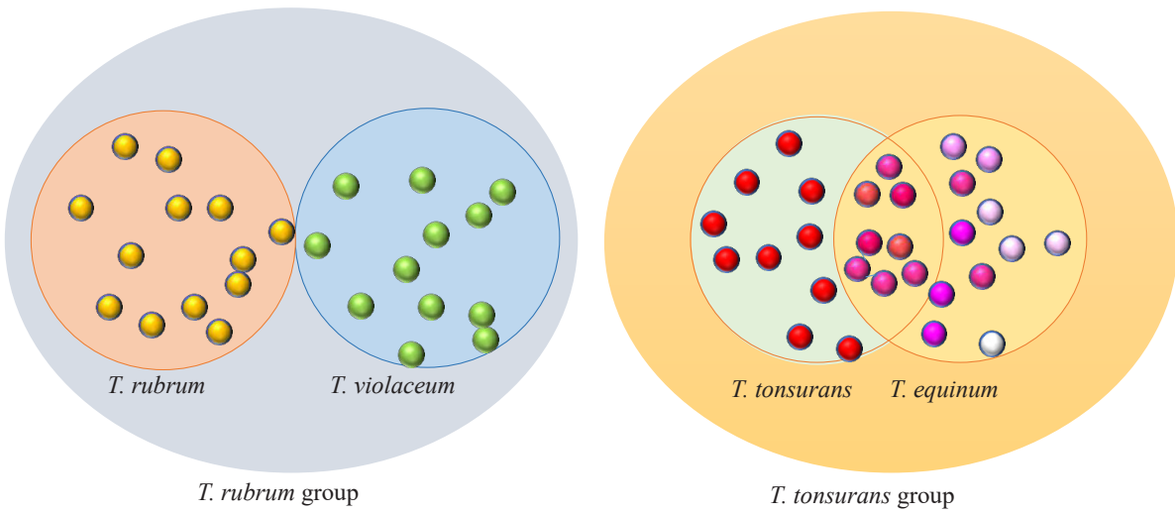

Figure 1. Two species groups, *T. rubrum* with *T. violaceum* and *T. tonsurans* with *T. equinum*. The members of *T. rubrum* group are considered to be different, whereas the separation of the other pair is doubtful, possibly involving only a single species.

and being endemic to Northern Africa and the Middle East. *Trichophyton soudanense* belongs to the latter group but is generally judged a synonym of *T. violaceum* (14). This set is compared to the *Trichophyton tonsurans* complex which comprises two lineages that are often regarded as synonymous (15): *T. tonsurans* and *T. equinum*. The former is an anthropophilic entity causing tinea capitis in humans, while its zoophilic counterpart *T. equinum* causes ringworm in horses but is also found in humans (3).

Our study successfully demonstrates a solution to a long existing technical challenge, i.e. the possibility to employ liquid chromatography coupled with ultra-high resolution Orbitrap mass spectrometry for microbial species identification. Massive quantities of fully resolved individual microbial proteins render Orbitrap mass spectrometry several orders of magnitude higher in sensitivity-specificity over than currently existing proteomic technologies. Subsequently this will set the stage to improve patient care significantly enabling microbial identification down to the strain level.

Materials and methods

Strains and growth conditions

Strains studied were acquired from the reference collection of Centraalbureau voor Schimmelcultures at the Westerdijk Fungal Biodiversity Institute (Table 1). Strains were part of a taxonomic study applying multilocus sequencing (3) and included (neo) type strains of synonymized species *Trichophyton raubitschekii, T. rubrum* var. *nigricans, T. fischeri, T. soudanense* and *T. violaceum* in the *T. rubrum* group, and *Trichophyton areolatum, T. floriforme, T. equinum* and *T. equinum* var. *autotrophicum* in the *T. tonsurans* group. Two strains of *T. interdigitale* were included as closest relatives of *T. tonsurans* serving as a marker of non-identity. Nine out of twelve strains in this group had variously been classified as either *T. tonsurans* or *T. equinum* (Table 1). Lyophilized or cryopreserved material was activated on Sabouraud's glucose agar plates (SGA; Oxoid, Thermo Scientific) and incubated at 24 °C for three weeks due to slow growth of *T. violaceum*.

Table 1. *Trichophyon* strains analyzed in this study.

Trichophyton strains	Taxonomy changes	Source	Clinical picture	Country	Gen Bank acc. No.
T. rubrum CBS 115314		human	onychomycosis	Greece	KT155714
T. rubrum CBS 100084 (T)	*T. raubitschekii*	human	skin	Canada	KT155667
T. rubrum CBS 100238	*T. rubrum var. nigicans*	human			KT155669
T. rubrum CBS 288.86	*T. fischeri*	contaminant		Canada	AJ270793
T. rubrum CBS 202.88	*T. raubitschekii*	human	tinea pedis	Canada	AJ270804
T. rubrum CBS 118892		human	onychomycosis	Germany	KT155731
T. violaceum CBS 120320		human	tinea capitis	Switzerland	KT155740
T. violaceum CBS 120316		human	tinea capitis	Switzerland	KT155737
T. violaceum CBS 201.88	*T. soudanense*	human	tinea faciei	Canada	KT310173
T. violaceum CBS 452.61	*T. soudanense*	human	endothirx and tinea capitis	Zaire	AJ270809
T. tonsurans CBS 285.30 (T)	*T. areolatum*	human	endrothrix	Argentina	KT155645
T. tonsurans CBS 318.31 (T)	*T. floriforme*	human			KT310170
T. tonsurans CBS 856.71	*T. equinum var equinum*	horse	hair	The Netherlands	KT310172
T. tonsurans CBS 127.97	*T. equinum, T equinum var equinum*	human	onychomycosis and tinea manuum	Finland	KT310169
T. tonsurans CBS 109033	*T. equinum*	horse	skin	Canada	KT155681
T. tonsurans CBS 112186		human		England	KT155688
T. equinum CBS 270.66 (NT)	*T. equinum var. equinum, T. tonsurans*	horse		USA	KT155643
T. equinum CBS 634.82	*T. equinum var. autotrophicum, T. tonsurans*	horse	tinea	New Zeland	KT310171
T. equinum CBS 100080 (T)	*T. equinum var. autotrophicum, T. tonsurans*	horse		New Zeland	KT155665
T. equinum CBS 112188	*T. tonsurans*	horse		England	EF043277
T. equinum CBS 112193	*T. tonsurans*	horse		England	KT155693
T. equinum CBS 112198	*T. tonsurans*	horse		England	EF043275
T. interdigitale CBS 119447		human	tinea capitis	Gabon	KT155733
T. interdigitale CBS 120318		human	tinea capitis	Switzerland	KT155738

T = type, NT = neotype. Data for taxonomy changes are all name changes recorded in CBS database (Previous nomenclatural changes for a particular strain).

DNA extraction, PCR and sequencing

Genomic DNA was extracted using Illumina's MasterPure™ DNA Purification Kit (Illumina) according to the manufacturer's protocol. rDNA ITS was sequenced using ITS5 and ITS4 primers under standard conditions (16). PCR products were purified with FastAP thermo sensitive alkaline phosphatase and shrimp alkaline phosphatase (Fermentas, Thermo Scientific). Sequencing reactions were done in 10 µL volumes using Thermo Scientific big dye terminator v.3.1 on a 3730XL instrument (Thermo Scientific). Sequences were deposited at NCBI GenBank (Table 1). Obtained sequences were manually edited and consensus sequences were aligned with MAFFT v. 6.850b with default settings (17). Identification was performed by querying sequences against NCBI GenBank and theWesterdijk Institute website (www.westerdijkinstitute.nl).

Protein extraction and purification

Protein extractions were performed on three biological replicates per strain. Briefly, approximately 5 mg biomass was harvested with a scalpel from a culture plate and transferred to a micro-vial (Eppendorf) with lysis buffer containing formic acid and acetonitrile (proprietary ratios). Cells were disrupted and then centrifuged for 1 minute at 14,000 rpm. The supernatant was then transferred to a new vial. Extracts were diluted to 10 % acetonitrile and were de-salted using Lab_in_a_Plate™ plates (Glygen Corp., USA). Equilibration, loading and washing steps were done according to the manufacturer's protocol with minor changes. Samples were eluted in 40 % acetonitrile with 0.1 % formic acid.

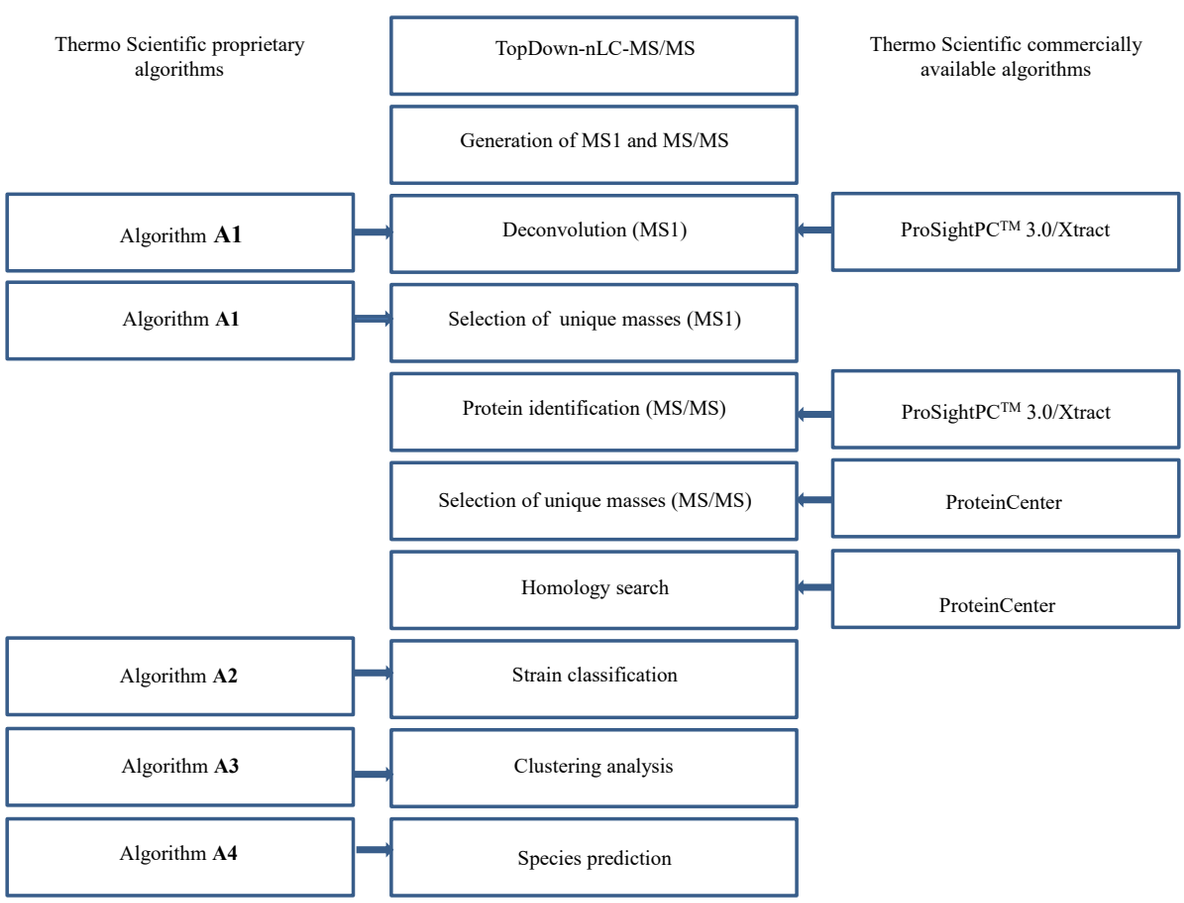

Figure 2. Overview of all Thermo Scientific algorithms employed in stepwise analysis of LC-MS/MS data.

LC-MS/MS analysis, data processing and identification

Chromatographic separation was done by injecting 8 µL of the protein extracts on a Thermo Scientific EASY-Spray Accucore™ C4 column, 15 cm × 75 µm ID, 2.6 µm particles and 150 Å pore size. Protein separation was achieved with one hour gradient starting with buffer A (0 % acetonitrile, 0.1 % formic acid) to 60 % of buffer B (60 % acetonitrile and 0.1 % formic acid) in 50 minutes, at a column temperature of 60 °C and a flow rate of 200 µL/min. The LC system was coupled with a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole mass spectrometer. Mass analysis was done with top-down method of 5 microscans, a scan range of 350–2000 Da, and loop counts for dd analysis being 15.

Algorithms used for further analysis of the acquired data are given in Fig. 2. Deconvolution of the mass spectra was performed via two algorithms. The first employed Thermo Fisher Scientific proprietary software (algorithm A1) to deconvolute raw spectra in m/z space into monoisotopic protein masses. An alternative approach was conducted via Thermo Scientific ProSightPC™ 3.0 for deconvolution of intact protein mass spectra and analysis of MS/MS fragment spectra (Thermo Scientific Xtract build-in). Subsequently, MS /MS fragment spectra were queried against a custom database obtained from Uniport (http://www.uniprot.org/) using ProSight PC™ 3.0, and containing amino acid sequences of the genera *Trichophyton, Microsporum, Epidermophyton* and *Arthroderma* (883,412 predicted proteoforms in total). The queries were performed as absolute mass search (considering disulfide linkages and Δm applied) in a specified intact mass window of 1000 Da; selected parameters were defined as 15 ppm fragment mass tolerance, acetylation and posttranslational modifications (PTM) applied as a criteria and cut-off expectation value (e-value) < 0.0001. Identified protein sequences with a confidence (e-value) score higher than 1.0×10^{-4} were further analyzed in the versatile custom sequence database and analysis software ProteinCenter. Using ProteinCenter the identified proteins were subjected to homology search using 80 % similarity cut off in attempt to find identical or similar sequences in other dermatophytes. Subsequently searches were constrained to 100 % homology level to clear redundancies.

Next to inferring species affiliations with identified protein sequences from MS/MS fragment spectra, we ran two additional analyses, to classify the strains and to predict the species where the analyzed strains belong. A yet unreported Thermo Fisher proprietary classification algorithm (algorithm A2) inferred the strain classification analysis. The prediction was repeated four times in order to establish variance between replicates. The resulting classification accuracy has values from 0 to 1, with 1 being all three replicates of a strain that were correctly identified in all four predictions.

Species prediction was performed with algorithm A4. The same data were used to establish statistical independence over the current taxonomic species affiliation, but constrained to a chosen reference strain to guide the species prediction. The analysis was conducted twice with different reference strains, each analysis with three iterations for the individual taxa. Two analyses were conducted with one or two reference strains. In the first analysis, one or two (neo) type strains or randomly chosen strains were used as reference strains to infer accuracy. Prior the second analysis, we performed clustering of 500 most consistently measured monoisotopic masses using a Thermo Scientific proprietary clustering algorithm A3 (data not shown). Clustering of strains with these monoisotopic masses revealed which strains have the highest number of shared masses. Based on this criterion, one or two of these strains were chosen as references. The latter step was required to avoid atypical selections.

Table 2. Number of obtained monoisotopic molecular weights by deconvolution of the MS1 spectra with algorithm 1 and ProSighPCTM 3.0. Variation between shared masses between all replicates for ProSight/Protein Centre data processing, results from different e-value/homology level cut-off criteria (See Material and Methods).

Strain	Algorithm 1 (A1)							ProSightPCTM 3.0							
	Total number of identified proteins			Statistics			Shared masses (Algorithm A1)	Total number of identified proteins			Statistics			Shared masses (ProSight)	Shared masses (Protein Centre)
	rep 1	rep 2	rep 3	AVG	SD	CV		rep 1	rep 2	rep 3	AVG	SD	CV		
T. rubrum CBS 115314	140	197	130	156	36	23	52	306	552	320	393	138	35	153	16
T. rubrum CBS 100084	182	214	162	186	26	14	88	392	373	320	362	37	10	195	21
T. rubrum CBS 100238	276	314	299	296	19	6	170	629	647	727	668	52	8	457	50
T. rubrum CBS 288.86	417	433	340	397	50	13	205	785	818	749	784	35	4	486	51
T. rubrum CBS 202.88	228	233	227	229	3	1	130	464	644	551	553	90	16	432	45
T. rubrum CBS 118892	346	311	367	341	28	8	195	675	655	810	713	84	12	523	53
T. violaceum CBS 120320	287	190	249	242	49	20	95	395	385	451	410	36	9	304	30
T. violaceum CBS 120316	218	323	277	273	53	19	147	383	591	536	503	108	21	309	39
T. violaceum CBS 201.88	264	257	286	269	15	6	160	697	711	664	691	24	3	513	56
T. violaceum CBS 452.61	394	380	338	371	29	8	218	535	775	680	663	121	18	313	32
T. tonsurans CBS 285.30	336	282	305	308	27	9	187	535	547	419	500	71	14	277	43
T. tonsurans CBS 318.31	153	170	126	150	22	15	81	290	313	227	277	45	16	202	28
T. tonsurans CBS 856.71	124	116	80	107	23	22	57	137	193	159	163	28	17	126	22
T. tonsurans CBS 127.97	345	327	337	336	9	3	193	456	441	454	450	8	2	340	50
T. tonsurans CBS 109033	322	350	308	327	21	7	148	459	340	607	469	134	29	241	43
T. tonsurans CBS 112186	313	251	225	263	45	17	119	494	473	353	440	76	17	262	42
T. equinum CBS 270.66	134	185	205	175	37	21	86	333	448	385	389	58	15	279	46
T. equinum CBS 634.82	274	313	262	283	27	9	162	297	399	290	329	61	19	231	32
T. equinum CBS 100080	382	396	365	381	16	4	222	479	550	648	559	85	15	329	62
T. equinum CBS 112188	204	256	265	242	33	14	116	486	483	523	497	22	4	325	48
T. equinum CBS 112193	184	225	177	195	26	13	103	368	434	350	384	44	12	266	73
T. equinum CBS 112198	242	145	243	210	56	27	91	420	387	421	409	19	5	300	29
T. interdigitale CBS 119447	395	387	450	411	34	8	259	683	612	697	664	46	7	474	71
T. interdigitale CBS 120318	305	304	292	300	7	2	165	605	568	497	557	55	10	369	54

rep = replicate, AVG = average, SD = standard deviation, CV = coefficient of variation.

Results

DNA-based identification

All strains had been identified prior to protein analysis using rDNA ITS as a barcode. Nucleotide sequence differences were established by separately aligning the two complexes. In the *T. rubrum* complex, *T. rubrum* strains differed from *T. violaceum* (including *T. soudanense*) with 4 SNPs at positions 167 (ITS1), 525, 543 and 544 (ITS2). Differences in the number of AT repeats at the end of ITS2 reported in the literature were not found to differ consistently in this alignment, and were therefore not taken into account. The six ITS sequences of *T. rubrum* were identical. In *T. violaceum,* CBS 452.61 denominated as *T. soudanense* was identical to remaining two *T .violaceum* strains, while the second *T. soudanense,* CBS 201.88 had a deletion of 36 bp, as reported in the literature. In the *T. tonsurans* / *T. equinum* group, the known C/T SNP was not distributed as expected between *T. tonsurans* and *T. equinum* strains; only CBS 318.31 had a C-SNP, while all remaining eleven strains, including the neotype of *T. equinum* CBS 270.66 had a T-SNP. The sequence of CBS 318.31 was identical to the neotype of *T. tonsurans* CBS 496.48. *Trichophyton interdigitale* differed from the *T. tonsurans / T. equinum* group by 9 nucleotides.

Deconvolution of raw mass spectra

Results from deconvoluting MS1 spectra employing a proprietary algorithm (A1) and ProSight PC™ 3.0 (Xtract) followed by processing MS/MS fragment spectra are summarized in Table 2. Total numbers of identified monoisotopic protein masses are given per replicate, with shared masses per strain, average number (AV), standard deviation (SD) and coefficients of variation (CV) representing biological variation. With algorithm A1, the lowest number of monoisotopic masses (52) was observed in *T. rubrum* CBS 115314 and the highest (259) in *T. interdigitale* CBS 119447. Standard deviations ranged between 3 and 53 in *T. rubrum* CBS 202.88 and *T. violaceum* CBS 120316, respectively. The CV was above 20 % for five strains, being the lowest (1 %) in *T. rubrum* CBS 118892 and the highest (27 %) in *T. equinum* CBS 112198.

Deconvolution employing ProSightPC™ 3.0 (Xtract) rendered the lowest number of protein masses (126) in *T. tonsurans* CBS 856.71 and the highest (523) in *T. rubrum* CBS 118892. Standard deviation ranged between 8 in *T. tonsurans* CBS 127.97 and 138 in and *T. rubrum* CBS 115314. The CV was 3 % in *T. violaceum* CBS 201.88 and highest with 35 % in *T. rubrum* CBS 115314. ProteinCenter analysis of protein masses obtained by ProSightPC™ 3.0 resulted in a total of 413 proteins, present in at least one replicate of one of the 24 strains at 100 % sequence homology. The number of identified proteins at 100 % homology level is given in Table 2 after post processing of initial ProSight results. The list of these proteins is given as supplementary material (ST1). In all subsequent analyses, only deconvoluted masses (algorithm A1) and/or identified proteins (ProSightPC™ 3.0) present in all three biological replicates are used.

Strain classification and species prediction

Monoisotopic masses obtained by A1 were further analyzed in order to select unique masses per strain, to classify the strains and to predict species affiliations. Strain classification performed on all replicates with four prediction runs is given in supplementary Table ST2. The highest classification accuracy (Table 3) is achieved with a score of one, while a no-call is defined as zero. The results indicate that

all strains affiliated to *Trichophyton rubrum, T. violaceum* and *T. interdigitale* were correctly classified with a classification accuracy of 1. In the *Trichophyton tonsurans / T. equinum* group, four strains, CBS 318.31, CBS 285.30, CBS 100080 and CBS 865.71 were correctly identified with the maximum classification accuracy. The remaining eight strains in this group were identified with a classification

Table 3. Strain classification obtained by algorithm A2.

Trichophyton strains	**CA (A2)**	**(#) strain unique masses (A1)**
T. rubrum CBS 115314	1	(1) 22,241.054
T. rubrum CBS 100084 (T)	1	(4) 18,812.048, 10,706.868, 7,384.616, 7,983.656
T. rubrum CBS 100238	1	(6) 7,480.315, 7,975.984, 22,046.173, 6,201.287, 21,707.120, 7,309.791
T. rubrum CBS 288.86	1	(8) 16,934.943, 9,368.722, 7,881.010, 11,873.910, 18,185.271, 7,779.975, 19,733.724, 11,861.896
T. rubrum CBS 202.88	1	(3) 5,437.895, 8,037.319, 5,227.340
T. rubrum CBS 118892	1	(3) 5,209.149, 12,397.609, 5,338.210
T. violaceum CBS 120320	1	(8) 9,852.837, 13,331.557, 5,301.844, 9,865.257, 5,073.543, 7,874.989, 10,851.584, 7,281.114
T. violaceum CBS 120316	1	(7) 6,067.292, 6,198.456, 7,139.672, 19,264.358, 5,147.600, 5,251.738, 12,669.770
T. violaceum CBS 201.88	1	(6) 6,660.335, 8,404.081, 9,832.479, 7,433.682, 9,363.639, 12,924.041
T. violaceum CBS 452.61	1	(7) 5,522.724, 12,042.842, 6,582.882, 6,500.904, 7,781.361, 20794.42548, 12339.29146
T. tonsurans CBS 285.30 (T)	1	(11) 11,388.283, 10,124.535, 23,933.015, 19,316.943, 9,897.097, 13,636.961, 5,183.505, 10,010.170, 15,833.425, 8,114.057, 20915.14524
T. tonsurans CBS 318.31 (T)	1	(5) 6,698.344, 8,927.547, 5,271.958, 7,300.539, 7,523.449
T. tonsurans CBS 856.71	1	(17) 5,169.601, 5,650.649, 5,918.855, 5,934.868, 6,686.769, 6,702.530, 7,005.805, 7,808.024, 8,007.802, 8,148.177, 8,199.929, 8,560.452, 8,915.246, 9,659.657, 9,717.965, 13,008.799, 17,112.085
T. tonsurans CBS 127.97	0,42	(1) 9,945.362
T. tonsurans CBS 109033	0,42	
T. tonsurans CBS 112186	0,92	
T. equinum CBS 270.66 (NT)	0,42	
T. equinum CBS 634.82	0,83	
T. equinum CBS 100080 (T)	1	(11) 11,196.118, 6,496.162, 14,082.605, 5,679.384, 7,750.926, 14,327.736, 6,955.962, 13,134.323, 7,118.017, 6,398.192, 6,105.138
T. equinum CBS 112188	0,67	
T. equinum CBS 112193	0,75	
T. equinum CBS 112198	0,67	
T. interdigitale CBS 119447	1	(16) 9,770.501, 9,659.784, 9,282.621, 5,373.858, 17,020.294, 11,145.854, 28,820.684, 10,022.215, 21,168.266, 21,271.700, 11,174.914, 19,542.778, 19,529.736, 21,354.18,9,908.134, 7,323.754
T. interdigitale CBS 120318	1	(7) 5,065.410, 5,091.356, 5,316.596, 6,488.034, 9,175.903, 13,247.418, 19,206.537

CA=coefficient of accuracy, A2=algorithm 2, A1=algorithm 1, (#) number of unique masses per strain

accuracy ranging from 0.42 to 0.92. Unique masses per strain were those masses present in all three replicates of a given strain and absent in all replicates from the remaining 24 strains.

Species prediction was performed using two independent analyses, each applying one or two reference strains (types or randomly chosen strains) for each cluster (Table 4). While selection of a single reference resulted in inconsistent species calls for both species complexes, adding another strain to the classifier improved classification accuracy by assessing proteome variability. With minor ambiguities, all strains in the *T. rubrum* complex were correctly identified. In the *T. tonsurans* complex, one-reference based prediction yielded a random spread of *T. tonsurans* and *T. equinum* calls. Addition of a second reference strain assigned only CBS 318.31 and CBS 285.30 to *T. tonsurans,* while the other 10 strains were assigned to *T. equinum* (exception in one call for CBS 109033, ST2).

A second analysis was performed with strains in each species with the highest number of shared masses. The references for *T. rubrum,* CBS 202.88 and CBS 118892 identified

Table 4. Species prediction of individual *Trichophyton* strains respectively and their references employed (references=individual mass spectra used relative to the taxon name identified by ITS sequencing).

***Trichophyton* strains**	**analysis 1**		**analysis 2**	
	reference 1	**references 1, 2**	**reference 1**	**references 1, 2**
T. rubrum CBS 115314	Tr Tr Tr	Tr Tr Tr	Tr Tr Tv	Tr Tr Tr
T. rubrum CBS 100084 (T)	reference	reference	Tr Tr Tr	Tr Tr Tr
T. rubrum CBS 1000238	Tr Tv Tv	reference	Tr Tr Tr	Tr Tr Tr
T. rubrum CBS 288.86	Tv Tv Tr	Tr Tv Tr	Tr Tr Tr	Tr Tr Tr
T. rubrum CBS 202.88	Tv Tr Tv	Tr Tr Tr	reference	reference
T. rubrum CBS 118892	Tv Tv Tv	Tr Tr Tr	Tr Tr Tr	reference
T. violaceum CBS 120320	reference	reference	Tv Tr Tv	Tv Tr Tv
T. violaceum CBS 120316	Tr Tr Tr	Tv Tv Tv	reference	reference
T. violaceum CBS 201	Tr Tr Tr	X Tv Tr	Tv Tv Tv	Tv Tr Tv
T. violaceum CBS 452.61	Tr Tr Tr	reference	Tv Tv Tv	reference
T. tonsurans CBS 285.30 (T)	reference	reference	Te Te Te	reference
T. tonsurans CBS 318.31 (T)	Tt Tt Tt	reference	reference	reference
T. tonsurans CBS 856.71	Te Te Te	Te Te Te	Te Te Te	Te Te Te
T. tonsurans CBS 127.97	Te Te Te	Te Te Te	Te Te Te	reference
T. tonsurans CBS 109033	Te Te Tt	Te Te Tt	Te Te Te	Te Te Te
T. tonsurans CBS 112186	Tt Tt Te	Te Te Te	Te Te Te	Te Te Te
T. equinum CBS 270.66 (NT)	Tt Tt Tt	reference	Tt Te Te	reference
T. equinum CBS 634.82	Te Te Te	Te Te Te	Te Te Te	Te Te Te
T. equinum CBS 100080 (T)	reference	reference	Te Te Te	Te Te Te
T. equinum CBS 112188	Tt Tt Te	Te Te Te	Te Te Te	Te Te Te
T. equinum CBS 112193	Tt Tt Tt	Te Te Te	Te Te Te	Te Te Te
T. equinum CBS 112198	Tt Tt Tt	Te Te Te	Te Te Te	Te Te Te
T. interdigitale CBS 119447	Te Te Te	Te Te Te	reference	reference
T. interdigitale CBS 120318	Te Tt Tt	Te Tt Tt	Ti Ti Ti	reference

Analysis 1: types, neotypes and/or randomly chosen strains were used as reference strains. Analysis 2: central strains with the highest number of shared masses (clustering analysis) were used as reference strains. T = type, NT = neotype, Tr = T. rubrum, Tv = *T. violaceum*, Tt = *T. tonsurans*, Te = *T. equinum,* Ti = *T. interdigitale*, X = no call. Colors visualize taxa.

correctly the remaining *T. rubrum* strains, while in *T. violaceum* addition of a second reference strain, CBS 452.61 to CBS 120316 did not improve the outcome. Species identifications for all strains primarily identified as *T. equinum* resulted consistently in *T. equinum,* but never *T. tonsurans*. Strains CBS 318.31 and CBS 285.30 were consistently assigned as *T. tonsurans.* The *T. interdigitale* strains matched with each other in the second analysis.

Selection of unique protein markers

Unique masses selected by one of the two or both algorithms, A1 and ProSightPC™ 3.0 are given in Table 5. Homologous proteins in other dermatophytes were matched using 80 % for global and 100 % for stringent sequence similarity filtering. In the *T. rubrum* group, both algorithms identified two out of six unique monoisotopic masses. Mass 6,391.358 was identified as hypothetical protein H100_08464 from *Trichophyton rubrum* MR850 (UNIPROT entry A0A022T914). Homology search in ProteinCenter revealed this protein in *T. equinum*, *T. interdigitale*, and *Microsporum gypseum* (*Nannizzia gypsea)* with monoisotopic mass 6,419.364 and one amino acid substitution. A second mass of 13,490.093 was identified as V-type ATPase G subunit from *Trichophyton rubrum* CBS 100081 (UNIPROT entry A0A022VE64). Homologs were found in *T. interdigitale* and *T. tonsurans* with monoisotopic masses 13,476.077 and 13,480.072, respectively. Four other proteins identified with ProSightPC™ 3.0 only were as follows: 7,553.051, identified as 40S ribosomal protein S28 from *Trichophyton rubrum* CBS 100081 (UNIPROT entry A0A022UXP6), 7,869.165, identified as hypothetical protein H106_04186 from *Trichophyton rubrum* CBS 735.88 (UNIPROT entry A0A028JNY6), 10,476.424, identified as hypothetical protein H102_06602 from *Trichophyton rubrum* CBS 100081 (UNIPROT entry A0A022UYS5) and 10,974.876, identified as hypothetical protein H107_03773 from *Trichophyton rubrum* CBS 202.88 (UNIPROT entry A0A023AI34).

Table 5. Unique masses per group and per species.

Group/species	Number of unique masses
T. rubrum / *T. violaceum*	(6) 6,391.358*, 13,490.093*, 7,553.051, 7869.165, 10,476.424, 10,974.876
T. tonsurans / *T. equinum* / *T. interdigitale*	(2) 7,883.18*, 10,450.732*
T. tonsurans / *T. equinum*	(3) 7,906.81*, 8,787.278, 11,464.894,
T. equinum / *T. interdigitale*	(3) 12,993.799, 15,859.410, 15,888.449
T. interdigitale	(5) 6,961.324, 9,014.567, 9,254.624, 9,481.539, 15,829.423
T. tonsurans	(2) 13,152.935, 17,474.951*

Unique masses identified by proprietary algorithm A1and/or ProSightPCTM 3.0. Proteins marked with * were identified by both algorithms.

In the *T. tonsurans* group (with *T. interdigitale* as species parameter), two unique masses were found and identified. One was a hypothetical protein TEQG_02912 from *Trichophyton equinum* CBS 127.97 (UNIPROT entry F2PPR0) with a mass of 7,883.18. This protein differs from its counterpart in the *T. rubrum* group (7,869.165) by one amino acid. The second one was hypothetical protein TEQG_00161 with a mass of 10,450.372 found in *T. equinum* CBS 127.97, (UNIPROT entry F2PGT9) differing from the *T. rubrum* protein (mass 10,476.424) by having one extra amino acid.

The *T. tonsurans* group (without *T. interdigitale*) had three unique masses, of which both algorithms identified a mass 7,906.81 as hypothetical protein TEQG_01010 from *Trichophyton equinum* CBS 127.97 (UNIPROT entry F2PJA2). The two other monoisotopic masses, 8,787.278 and 11,464.894 could not be identified by ProSightPC™ 3.0. *Trichophyton equinum* and *T. interdigitale* share three masses, 12,993.799, 15,859.411 and 15,888.449. There were no entries for these masses in ProSightPC™ 3.0. In contrast, *T. tonsurans* did not have masses in common with *T. interdigitale,* implying that *T. interdigitale* is likely more close phylogenetically to *T. equinum* than to *T. tonsurans*.

At the species level, no unique masses were found defining *T. rubrum*, *T. violaceum*, *T. equinum* or *T. tonsurans*. In contrast, *T. interdigitale* had five unique masses (6,961.324, 9,014.567, 9,254.624, 9,481.539, 15,829.423), none of which could be matched to any protein sequence predicted from the corresponding genomes. For *T. tonsurans,* as derived from the second species prediction approach, the two strains CBS 318.31 and CBS 285.30 affiliated to *T. tonsurans* share two unique masses: 13,152.935, found by algorithm A1 and 17,474.951 found by both algorithms, identified as hypothetical protein TESG_03051 from *T. tonsurans* CBS 112818 (UNIPROT entry F2RWA2).

Discussion

In this study, we evaluated the resolution power of LC-MS/MS as a novel method to delimit clinically relevant filamentous fungi, with two groups of dermatophytes, each containing two very closely related species as a model set. Nucleic acid based approaches like rDNA ITS sequence data are used as a gold standard, as this gene is judged optimal for dermatophyte diagnostics (3).

Separation of species within both groups is problematic and highly controversial. On the basis of molecular data, *Trichophyton tonsurans* (on humans) and *T. equinum* (on horses) had been regarded as synonyms (15). Matruchot and Dassonville (18) already reported transmission from horse to human in their original description of *T. equinum*. In the present study, two of the analyzed strains were used that had been transmitted from horse to human: CBS 127.97 (19) and CBS 270.66 (20). Woodgyer et al. (21) distinguished the species by a T/C SNP in ITS1 (C nucleotide in *T. tonsurans*, T nucleotide in *T. equinum*) and Chollet et al. (22) listed some phenotypic differences. We were unable to find correspondence between these criteria among our strains using the intact protein-based approach described herein. All but one (CBS 318.31) strains listed as *T. tonsurans* had the *T. equinum*-associated T nucleotide, which was also present in all six *T. equinum* strains. To verify the validity of this SNP in a larger dataset we randomly selected 64 strains from the CBS collection (data not shown). All *T. equinum* from horse had a T nucleotide, but 35 % of the *T. tonsurans* strains from humans had the same T nucleotide (data not shown). De Hoog et al. (3) were also unable to distinguish the two species using additional genes. More detailed patient and phenotypic information is necessary to establish whether *T. equinum* is a separate species at all. Using MALDI-TOF, Nenoff et al. (8) and De Respinis et al. (4) distinguished *T. tonsurans* and *T. equinum* but the authors did not present on which ground they denominated the strains as *T. tonsurans* or *T. equinum.* In our study, discrimination at strain level resulted in identification of 4 out of 12 strains of the *T. tonsurans* lineage. The remaining eight strains formed two clusters with overlapping proteomes: cluster 1 with CBS 642.82, CBS 127.97 and CBS 109033, and cluster 2 with two subclusters CBS 270.66 (overlapping with 112188), CBS 112198 (overlapping with 112186) and CBS 112193 (see ST2). In Fig. 1, these two clusters would be placed in the intersection of the two species clouds. Note that both clusters have strains isolated from both humans and horses, which contradicts the hypothesis of host-

based distinction in two species. In the species prediction analysis (Table 4) only CBS 318.31 and CBS 285.30 were affiliated as *T. tonsurans*, which is not in concordance with a T/C SNP for CBS 285.30. So far, typing strains within the *T. tonsurans / T. equinum* species complex appears to be challenging (pending method improvements) due to insufficiently resolved taxonomic definitions of known reference strains, which is likely due to conspecificity.

In MALDI-TOF analyses of De Respinis et al. (4) some *T. tonsurans* spectra were misidentified as *T. interdigitale*. Calderaro et al. (23) noted the same misidentification before the supplementation to Bruker's BioTyper database. With nine nucleotides difference in the ITS region, *T. interdigitale* should be easily distinguishable from the *T. tonsurans / T. equinum* complex. Separation was confirmed in all our analyses; with strain classification accuracies of 1, five unique species masses (Table 5) and their clustering as a distinct group in species prediction analysis (Table 4).

Analyses of the *T. rubrum* group were in concordance with previous findings. According to Gräser et al. (14), the *T. rubrum* group comprises only two anthropophilic species, *T. rubrum* and *T. violaceum*, the latter species with *T. soudanense* as a probable mutant and prevalently causing tinea capitis. *Trichophyton violaceum* is endemic in Africa (14, 24), while *T. rubrum is* cosmopolitan. Microsatellite analysis has revealed that *T. violaceum* is more variable than *T. rubrum*, with some strains being closer to *T. rubrum* than the others (25). *Trichophyton rubrum* and *T. violaceum* are morphologically very different, but similar in their DNA profiles. Our analyzed strains differed in four positions in ITS (data not shown). MALDI-TOF analyses frequently proved to be unable to separate the two species (4, 7, 8, 11). Summarized misidentifications and/or unreliable identifications of *T. tonsurans* (misidentified with *T. rubrum* and *vice versa*), *T. violaceum* and *T. soudanense* were recently reported by Sanguinetti and Posteraro (26). In the newest evaluation study of the VITEK v3.0 system for the identification of filamentous fungi (27), *Trichophyton* species were regarded as particularly problematic, with *T. interdigitale*, *T. tonsurans*, *T. violaceum* having the success percentage of 97 %, 91 % and 41 %, respectively, in the first attempt.

With LC-MS/MS, discrimination at the strain level was achieved with all six *T. rubrum* and four *T. violaceum* strains classified with classification analysis of 1. In this analysis, optimal species association was achieved with CBS 118892 as reference for *T. rubrum* and CBS 120316 as reference for *T. violaceum.* Notably, taxonomic types may be located eccentrically in the species cloud and thus provide less optimal results. Our analysis showed that *T. violaceum* strain CBS 120320 shares some features with *T. rubrum* (one call as *T. rubrum*, Table 4). Both strains denominated in the CBS collection as *T. soudanense*, CBS 452.61 and CBS 201.88 were affiliated to *T. violaceum*, fitting the ITS data. Interestingly, the only strain with 36 bp deletion, CBS 201.88, had one nonsense and one *T. violaceum* call in the first species prediction analysis (Table 4).

Conclusions

Whole protein top-down LC-MS/MS analysis has significant diagnostic potential because of its analytical performance level being higher than MALDI-TOF, particularly below the species level, i.e. at lineage or strain level. The accurate detection of protein masses, separation of high numbers of individual proteins and detection of single amino-acid exchanges are responsible for the high performance. The proprietary Thermo Scientific algorithms A2, A3 and A4 showed a potential to recognize individual strains, which can be applied in epidemics or outbreak scenarios. Detailed studies are however required since the choice of reference strains is crucial for appropriate species affiliation, as routine selection of taxonomic types may not provide

optimal results. Species limits and species variability in dermatophytes, which were classically distinguished on the basis of clinical and phenotypic criteria, have to be newly defined in order to develop reliable and predictive taxonomy and meaningful diagnostics tools.

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Conflict of interests

The authors Joanna Freeke, J. Benjamin Stielow, Ping Yip, James L. Stephenson and Azadeh Jamalian Nasrabi declare competing financial interest and are employees of Thermo Fisher Scientific.

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ST1. A list of identified proteins found in at least one replicate in at least one of the 24 strains.

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
1	2858,46	Full=83 kDa hypersensitivity protein; Short=Protein IV	Trichophyton tonsurans	P80514
2	5024,624	hypothetical protein H101_08178, partial	Trichophyton interdigitale H6	A0A022U3H1
3	5950,082	hypothetical protein TRV_01005	Trichophyton verrucosum HKI 0517	D4D1R2
4	5976,042	hypothetical protein TERG_03909	Trichophyton rubrum CBS 118892	F2SPB0
5	6082,104	hypothetical protein H109_03307	Trichophyton interdigitale MR816	A0A059JAQ7
6	6111,297	TOM complex component Tom7	Trichophyton equinum CBS 127.97	F2PV03
7	6249,552	60S ribosomal protein L39	Trichophyton soudanense CBS 452.61	A0A022Y8Q8
8	6374,187	hypothetical protein H104_04119	Trichophyton rubrum CBS 289.86	A0A022WZD0
9	**6391,358**	**hypothetical protein H100_08464**	**Trichophyton rubrum MR850**	**A0A022T914**
10	6419,364	hypothetical protein TEQG_08592	Trichophyton equinum CBS 127.97	F2PIW8
11	6431,245	hypothetical protein TERG_03909	Trichophyton rubrum CBS 118892	F2SPB0
12	6491,121	hypothetical protein MGYG_03527	Nannizzia gypsea CBS 118893	E4USF6
13	6642,338	40S ribosomal protein S29	Trichophyton rubrum MR1459	A0A022ZCH4
14	6657,771	ribosomal protein L33	Trichophyton rubrum CBS 100081	A0A022V366
15	6673,766	mitochondrial 54S ribosomal protein YmL39	Arthroderma otae CBS 113480	C5FQE6
16	6823,45	hypothetical protein TERG_11971	Trichophyton rubrum CBS 118892	A0A080WK51
17	6934,826	hypothetical protein H102_01912	Trichophyton rubrum CBS 100081	A0A022VEY7
18	7025,632	hypothetical protein MGYG_08779	Nannizzia gypsea CBS 118893	E4V6Z3
19	7164,456	hypothetical protein H103_05209	Trichophyton rubrum CBS 288.86	A0A022W0B9
20	7294,897	H/ACA ribonucleoprotein complex subunit 3	Trichophyton interdigitale MR816	A0A059JAQ0
21	7310,892	H/ACA ribonucleoprotein complex subunit 3	Arthroderma otae CBS 113480	C5FVJ5
22	7320,715	hypothetical protein TESG_03327	Trichophyton tonsurans CBS 112818	F2RX14
23	7334,73	hypothetical protein H109_04377	Trichophyton interdigitale MR816	A0A059J8G2
24	7336,71	hypothetical protein H106_05993	Trichophyton rubrum CBS 735.88	A0A028JJJ7
25	7350,611	hypothetical protein TESG_05851	Trichophyton tonsurans CBS 112818	F2S4H9
26	7370,694	hypothetical protein H105_06170	Trichophyton soudanense CBS 452.61	A0A022XMJ5
27	7420,67	hypothetical protein H112_04983	Trichophyton rubrum D6	A0A059JZF8
28	7422,632	hypothetical protein TEQG_04554	Trichophyton equinum CBS 127.97	F2PUH7
29	7471,856	Mitotic-spindle organizing protein 1	Trichophyton interdigitale H6	A0A022U716
30	7485,872	hypothetical protein MGYG_08659	Nannizzia gypsea CBS 118893	E4V6L8
31	7499,887	Mitotic-spindle organizing protein 1	Trichophyton rubrum CBS 289.86	A0A022WVV9
32	7549,887	40S ribosomal protein S29	Trichophyton equinum CBS 127.97	F2PHG7
33	**7553,051**	**40S ribosomal protein S28**	**Trichophyton rubrum CBS 100081**	**A0A022UXP6**
34	7569,045	40S ribosomal protein S28	Trichophyton interdigitale MR816	A0A059J6B3
35	7593,88	hypothetical protein H109_01378	Trichophyton interdigitale MR816	A0A059JG54
36	7647,822	hypothetical protein TEQG_05605	Trichophyton equinum CBS 127.97	F2PXI9
37	7690,933	hypothetical protein H107_02000	Trichophyton rubrum CBS 202.88	A0A023AQ26
38	7695,827	hypothetical protein H103_00600	Trichophyton rubrum CBS 288.86	A0A022WGL4
39	7708,907	hypothetical protein TEQG_00547	Trichophyton equinum CBS 127.97	F2PHT5
40	7755,104	protein translocase SEC61 complex gamma subunit	Trichophyton rubrum CBS 202.88	A0A023A6D6

ST1. (Continued)

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
41	7755,104	conserved hypothetical protein	Arthroderma otae CBS 113480	C5FZC5
42	7776,126	Non-histone chromosomal protein 6	Trichophyton interdigitale H6	A0A022U2R8
43	7828,147	hypothetical protein MGYG_08266	Nannizzia gypsea CBS 118893	E4V672
44	7831,146	hypothetical protein H100_03016	Trichophyton rubrum MR850	A0A022TSI8
45	**7869,165**	**hypothetical protein H106_04186**	**Trichophyton rubrum CBS 735.88**	**A0A028JNY6**
46	7876,799	hypothetical protein H109_05871	Trichophyton interdigitale MR816	A0A059J412
47	**7883,18**	**hypothetical protein TEQG_02912**	**Trichophyton equinum CBS 127.97**	**F2PPR0**
48	**7906,81**	**hypothetical protein TEQG_01010**	**Trichophyton equinum CBS 127.97**	**F2PJA2**
49	7911,063	hypothetical protein H105_06878	Trichophyton soudanense CBS 452.61	A0A022XJV4
50	7936,903	hypothetical protein H100_08883	Trichophyton rubrum MR850	A0A022T7A3
51	7995,084	hypothetical protein H103_06623	Trichophyton rubrum CBS 288.86	A0A022VV02
52	8132,933	hypothetical protein H101_07725	Trichophyton interdigitale H6	A0A022U460
53	8147,908	hypothetical protein TERG_02953	Trichophyton rubrum CBS 118892	F2SLT9
54	8262,385	Nop10 family nucleolar RNA-binding protein	Trichophyton tonsurans CBS 112818	F2RN39
55	8349,431	conserved hypothetical protein	Arthroderma otae CBS 113480	C5G0N6
56	8500,393	hypothetical protein H106_03055	Trichophyton rubrum CBS 735.88	A0A028JU11
57	8526,187	metallothionein-I transcription activator	Trichophyton rubrum CBS 118892	F2SXL9
58	8526,187	metallothionein-I transcription activator	Trichophyton tonsurans CBS 112818	F2S805
59	8546,188	hypothetical protein H105_08314	Trichophyton soudanense CBS 452.61	A0A022XEU8
60	8560,203	hypothetical protein H113_08378	Trichophyton rubrum MR1459	A0A022Z5R6
61	8655,356	hypothetical protein TERG_08213	Trichophyton rubrum CBS 118892	F2T075
62	8677,222	hypothetical protein TEQG_05411	Trichophyton equinum CBS 127.97	F2PWY9
63	8690,217	hypothetical protein H109_00201	Trichophyton interdigitale MR816	A0A059JKQ0
64	8704,232	hypothetical protein H102_00282	Trichophyton rubrum CBS 100081	A0A022VLB6
65	8750,411	ribosomal protein S21e	Trichophyton tonsurans CBS 112818	F2S3S0
66	8784,538	hypothetical protein TESG_08443	Trichophyton tonsurans CBS 112818	F2RYS1
67	8840,68	hypothetical protein TESG_06208	Trichophyton tonsurans CBS 112818	F2S5J5
68	8840,694	Small nuclear ribonucleoprotein SmG	Trichophyton rubrum CBS 118892	F2SIV7
69	8908,57	40S ribosomal protein S27	Trichophyton rubrum CBS 289.86	A0A022WNF6
70	8918,592	40S ribosomal protein S27	Arthroderma otae CBS 113480	C5FZK4
71	8963,868	60S ribosomal protein, partial	Trichophyton rubrum MR1459	A0A022ZRD6
72	9011,974	hypothetical protein H113_05862	Trichophyton rubrum MR1459	A0A022ZFB8
73	9067,354	hypothetical protein H104_00893	Trichophyton rubrum CBS 289.86	A0A022XAF9
74	9130,469	hypothetical protein H106_08183	Trichophyton rubrum CBS 735.88	A0A028JB47
75	9140,068	hypothetical protein H103_05821	Trichophyton rubrum CBS 288.86	A0A022VZ71
76	9232,089	hypothetical protein H110_06181	Trichophyton rubrum MR1448	A0A022YGY3
77	9244,486	hypothetical protein H109_03933	Trichophyton interdigitale MR816	A0A059J8L9
78	9246,141	60s ribosomal protein l38	Trichophyton equinum CBS 127.97	F2PW41
79	9256,941	60S ribosomal protein	Trichophyton rubrum MR1459	A0A022ZSJ9
80	9348,979	Cytochrome b5	Trichophyton equinum CBS 127.97	F2PP14
81	9350,94	cytochrome b5, putative	Trichophyton benhamiae CBS 112371	D4AU60
82	9376,985	hypothetical protein H101_07638	Trichophyton interdigitale H6	A0A022U4D1
83	9377,01	cytochrome b5, putative	Trichophyton verrucosum HKI 0517	D4DE89

ST1. (Continued)

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
84	9561,734	hypothetical protein H110_00692	Trichophyton rubrum MR1448	A0A022Z154
85	9577,782	hypothetical protein TEQG_08164	Trichophyton equinum CBS 127.97	F2Q4Z7
86	9583,833	40S ribosomal protein S21	Trichophyton rubrum CBS 735.88	A0A028JYC2
87	9591,983	hypothetical protein H100_06193	Trichophyton rubrum MR850	A0A022TGZ4
88	9635,788	Heat shock protein Awh11, putative	Trichophyton verrucosum HKI 0517	D4D992
89	9635,788	Heat shock protein Awh11, putative	Trichophyton benhamiae CBS 112371	D4ATB9
90	9642,968	hypothetical protein H112_01581	Trichophyton rubrum D6	A0A059KBL2
91	9665,799	hypothetical protein H109_06473	Trichophyton interdigitale MR816	A0A059J136
92	9681,793	hypothetical protein H112_08565	Trichophyton rubrum D6	A0A059JLR6
93	9811,563	conidiation-specific protein Con-10, putative	Trichophyton verrucosum HKI 0517	D4DAE6
94	9839,594	conidiation-specific protein Con-10, putative	Trichophyton benhamiae CBS 112371	D4AUM8
95	9853,717	conserved hypothetical protein	Arthroderma otae CBS 113480	C5FW50
96	9859,529	Conidiation-specific protein 10	Trichophyton interdigitale MR816	A0A059J9Z7
97	9873,545	hypothetical protein TEQG_08697	Trichophyton equinum CBS 127.97	F2PUC7
98	9876,732	hypothetical protein TRV_05378	Trichophyton verrucosum HKI 0517	D4DE14
99	9890,062	hypothetical protein H104_03541	Trichophyton rubrum CBS 289.86	A0A022X1B9
100	9898,956	hypothetical protein TESG_00720	Trichophyton tonsurans CBS 112818	F2RPB1
101	9906,743	hypothetical protein H103_04223	Trichophyton rubrum CBS 288.86	A0A022W3V7
102	9934,737	hypothetical protein H109_03021	Trichophyton interdigitale MR816	A0A059JBF5
103	10101,517	hypothetical protein H109_02823	Trichophyton interdigitale MR816	A0A059JCG1
104	10105,512	hypothetical protein H102_03915	Trichophyton rubrum CBS 100081	A0A022V8Q4
105	10115,532	hypothetical protein TESG_07549	Trichophyton tonsurans CBS 112818	F2S9I3
106	10119,491	hypothetical protein MGYG_03413	Nannizzia gypsea CBS 118893	E4UNF9
107	10145,306	40S ribosomal protein S27	Nannizzia gypsea CBS 118893	E4UWP1
108	10189,469	60S ribosomal protein L43	Nannizzia gypsea CBS 118893	E4V424
109	10205,464	60S ribosomal protein L43	Trichophyton rubrum D6	A0A059K219
110	10219,479	60S ribosomal protein L43	Trichophyton interdigitale MR816	A0A059JBX3
111	10327,038	hypothetical protein H107_06055	Trichophyton rubrum CBS 202.88	A0A023A940
112	10371,184	hypothetical protein TEQG_03178	Trichophyton equinum CBS 127.97	F2PQH7
113	10387,179	hypothetical protein H103_04689	Trichophyton rubrum CBS 288.86	A0A022W1W8
114	10425,134	hypothetical protein H104_08266	Trichophyton rubrum CBS 289.86	A0A022WKH6
115	10425,134	NADH-ubiquinone oxidoreductase B18 subunit	Trichophyton tonsurans CBS 112818	F2S0M1
116	**10450,372**	**hypothetical protein TEQG_00161**	**Trichophyton equinum CBS 127.97**	**F2PGT9**
117	**10476,424**	**hypothetical protein H102_06602**	**Trichophyton rubrum CBS 100081**	**A0A022UYS5**
118	10478,379	hypothetical protein TRV_00578	Trichophyton verrucosum HKI 0517	D4D0I3
119	10519,148	ribosomal protein A1	Trichophyton tonsurans CBS 112818	F2RZP6
120	10586,893	40S ribosomal protein S25	Nannizzia gypsea CBS 118893	E4V2D3
121	10588,872	40S ribosomal protein S25	Arthroderma otae CBS 113480	C5FBE7
122	10616,939	40S ribosomal protein S25	Trichophyton equinum CBS 127.97	F2Q2F3
123	10678,814	LSM domain-containing protein	Nannizzia gypsea CBS 118893	E4V3J8
124	10736,82	hypothetical protein H113_03339	Trichophyton rubrum MR1459	A0A022ZNK2

ST1. (Continued)

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
125	10776,231	NADH:ubiquinone oxidoreductase kDa subunit	Nannizzia gypsea CBS 118893	E5R3T8
126	10782,769	hypothetical protein H107_03434	Trichophyton rubrum CBS 202.88	A0A023AIQ4
127	10818,278	hypothetical protein H112_07904	Trichophyton rubrum D6	A0A059JNV3
128	10838,562	conserved hypothetical protein	Arthroderma otae CBS 113480	C5FUI5
129	10858,644	hypothetical protein H109_07557	Trichophyton interdigitale MR816	A0A059IY00
130	10861,284	NADH:ubiquinone oxidoreductase 11.5kD subunit	Trichophyton tonsurans CBS 112818	F2S2S4
131	10868,487	Ubiquinol-cytochrome c reductase complex	Trichophyton equinum CBS 127.97	F2Q5B3
132	10888,654	hypothetical protein H103_00309	Trichophyton rubrum CBS 288.86	A0A022WG40
133	10907,128	Cytochrome c oxidase polypeptide vib	Nannizzia gypsea CBS 118893	E4UQM5
134	10930,585	glutaredoxin	Trichophyton rubrum CBS 289.86	A0A022WLE1
135	**10974,876**	**hypothetical protein H107_03773**	**Trichophyton rubrum CBS 202.88**	**A0A023AI34**
136	10986,67	glutaredoxin	Trichophyton interdigitale MR816	A0A059J445
137	11002,907	ATP synthase subunit F	Trichophyton tonsurans CBS 112818	F2S384
138	11003,413	hypothetical protein MGYG_01517	Nannizzia gypsea CBS 118893	E5R1A0
139	11003,413	60S acidic ribosomal protein P1	Trichophyton interdigitale MR816	A0A059JID4
140	11004,887	hypothetical protein H101_03632	Trichophyton interdigitale H6	A0A022UFQ5
141	11017,429	hypothetical protein ARB_00018	Trichophyton benhamiae CBS 112371	D4AV09
142	11025,476	hypothetical protein H104_03149	Trichophyton rubrum CBS 289.86	A0A022X2C4
143	11033,424	60S acidic ribosomal protein P1	Arthroderma otae CBS 113480	C5FCM0
144	11044,954	ATP synthase f chain	Arthroderma otae CBS 113480	C5FM10
145	11051,241	hypothetical protein H104_05804	Trichophyton rubrum CBS 289.86	A0A022WSK7
146	11107,405	hypothetical protein TRV_07083	Trichophyton verrucosum HKI 0517	D4DIS2
147	11128,031	hypothetical protein H104_00386	Trichophyton rubrum CBS 289.86	A0A022XBP6
148	11134,417	60S acidic ribosomal protein P2	Trichophyton interdigitale H6	A0A022UDH1
149	11134,417	hypothetical protein ARB_06463	Trichophyton benhamiae CBS 112371	D4AQF6
150	11161,428	60S acidic ribosomal protein P2	Trichophyton soudanense CBS 452.61	A0A022XKK8
151	11164,428	60S acidic ribosomal protein P2	Trichophyton tonsurans CBS 112818	F2RX80
152	11181,035	Mitochondrial heat shock protein Hsp10	Trichophyton benhamiae CBS 112371	D4B2T3
153	11343,367	Histone	Trichophyton rubrum CBS 100081	A0A022V2V7
154	11373,378	Histone	Trichophyton rubrum D6	A0A059JV68
155	11401,486	Cytochrome c oxidase polypeptide vib	Trichophyton equinum CBS 127.97	F2PLP7
156	11413,653	hypothetical protein H109_00222	Trichophyton interdigitale MR816	A0A059JJW1
157	11416,061	60S ribosomal protein	Trichophyton rubrum MR850	A0A022TUU8
158	11446,07	60S ribosomal protein L30	Arthroderma otae CBS 113480	C5FTF5
159	11491,878	Acyl CoA binding protein family protein	Trichophyton benhamiae CBS 112371	D4AZ00
160	11495,801	hypothetical protein H107_03639	Trichophyton rubrum CBS 202.88	A0A023AI70
161	11511,194	hypothetical protein H109_06884	Trichophyton interdigitale MR816	A0A059J064
162	11530,61	hypothetical protein H105_00298	Trichophyton soudanense CBS 452.61	A0A022Y7U0
163	11537,884	Acyl CoA binding protein family	Trichophyton verrucosum HKI 0517	D4D796
164	11537,884	hypothetical protein H109_02564	Trichophyton interdigitale MR816	A0A059JD51
165	11553,915	hypothetical protein TEQG_01139	Trichophyton equinum CBS 127.97	F2PJN2

ST1. (Continued)

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
166	11604,74	CipC protein	Arthroderma otae CBS 113480	C5FU43
167	11678,783	eukaryotic translation initiation factor 5A	Trichophyton equinum CBS 127.97	F2PJS6
168	11690,619	60S ribosomal protein L36	Trichophyton rubrum CBS 735.88	A0A028JH03
169	11706,614	60S ribosomal protein L36	Nannizzia gypsea CBS 118893	E4UW88
170	11710,05	hypothetical protein H113_05269	Trichophyton rubrum MR1459	A0A022ZH00
171	11718,65	60S ribosomal protein L36	Trichophyton verrucosum HKI 0517	D4D6R6
172	11727,004	Non-histone chromosomal protein 6	Trichophyton rubrum MR1448	A0A022YXQ9
173	11771,03	Nucleosome binding protein	Trichophyton tonsurans CBS 112818	F2S7D0
174	11778,896	hypothetical protein H110_08751	Trichophyton rubrum MR1448	A0A022Y9X9
175	11842,063	hypothetical protein TESG_00838	Trichophyton tonsurans CBS 112818	F2RPQ6
176	11843,046	hypothetical protein H109_06227	Trichophyton interdigitale MR816	A0A059J2X1
177	11849,368	hypothetical protein H101_08083, partial	Trichophyton interdigitale H6	A0A022U338
178	11872,059	NADH-ubiquinone oxidoreductase subunit	Nannizzia gypsea CBS 118893	E4UVI5
179	11898,425	60S ribosomal protein L33	Arthroderma otae CBS 113480	C5FQK6
180	11899,141	hypothetical protein H101_01833	Trichophyton interdigitale H6	A0A022UMC2
181	11906,043	hypothetical protein ARB_02394	Trichophyton benhamiae CBS 112371	D4B1R3
182	12009,764	hypothetical protein TRV_02149	Trichophyton verrucosum HKI 0517	D4D4Y1
183	12024,738	hypothetical protein ARB_00575	Trichophyton benhamiae CBS 112371	D4AWK9
184	12087,505	LYR family protein	Trichophyton equinum CBS 127.97	F2PL66
185	12156,671	chaperonin GroS	Nannizzia gypsea CBS 118893	E5R1X1
186	12179,059	hypothetical protein H112_01497	Trichophyton rubrum D6	A0A059KAG9
187	12193,109	cytochrome c	Trichophyton rubrum CBS 118892	A0A080WFY2
188	12297,663	60S ribosomal protein L33-A	Trichophyton rubrum CBS 289.86	A0A022WV96
189	12298,647	60S ribosomal protein L33-A	Trichophyton interdigitale H6	A0A022ULJ8
190	12302,329	hypothetical protein TRV_00851	Trichophyton verrucosum HKI 0517	D4D1A3
191	12335,315	hypothetical protein TEQG_06872	Trichophyton equinum CBS 127.97	F2Q1V3
192	12348,616	chaperonin 10 Kd subunit	Trichophyton tonsurans CBS 112818	F2RWD9
193	12526,289	hypothetical protein TRV_07039	Trichophyton verrucosum HKI 0517	D4DIM9
194	12543,25	hypothetical protein H101_01020	Trichophyton interdigitale H6	A0A022UPB3
195	12661,688	Ribonucleoprotein-associated protein	Trichophyton equinum CBS 127.97	F2PK61
196	12753,907	ribosomal L15	Nannizzia gypsea CBS 118893	E4V4H5
197	12824,541	hypothetical protein TESG_01520	Trichophyton tonsurans CBS 112818	F2RRT2
198	13043,89	translation initiation factor SUI1	Trichophyton rubrum D6	A0A059JSM3
199	13048,586	progesterone binding protein	Nannizzia gypsea CBS 118893	E5R411
200	13075,634	progesterone binding protein	Trichophyton tonsurans CBS 112818	F2RRT7
201	13102,645	hypothetical protein H112_04837	Trichophyton rubrum D6	A0A059K064
202	13143,114	hypothetical protein TESG_02806	Trichophyton tonsurans CBS 112818	F2RVH0
203	13158,933	LEA domain protein	Trichophyton verrucosum HKI 0517	D4D0M9
204	13159,815	hypothetical protein H107_05463	Trichophyton rubrum CBS 202.88	A0A023ACA9
205	13163,717	hypothetical protein H106_00948	Trichophyton rubrum CBS 735.88	A0A028JZH6
206	13216,942	hypothetical protein H101_02845	Trichophyton interdigitale H6	A0A022UIA8
207	13257,254	40S ribosomal protein S20	Trichophyton rubrum MR1448	A0A022YE10
208	13263,806	hypothetical protein TEQG_02091	Trichophyton equinum CBS 127.97	F2PMF7

ST1. (Continued)

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
209	13476,077	V-type ATPase, G subunit	Trichophyton interdigitale H6	A0A022UJR0
210	13480,072	vacuolar ATPase	Trichophyton tonsurans CBS 112818	F2S9B6
211	**13490,093**	**V-type ATPase, G subunit**	**Trichophyton rubrum CBS 100081**	**A0A022VE64**
212	13510,123	hypothetical protein H101_07032	Trichophyton interdigitale H6	A0A022U5Y4
213	13636,525	60S ribosomal protein L34-B	Nannizzia gypsea CBS 118893	E4V0E3
214	13647,541	60S ribosomal protein L34	Arthroderma otae CBS 113480	C5FT72
215	13661,233	small nucleolar ribonucleoprotein SNU13	Arthroderma otae CBS 113480	C5FM70
216	13663,536	hypothetical protein H112_02454	Trichophyton rubrum D6	A0A059K739
217	13686,776	hypothetical protein TESG_00525	Trichophyton tonsurans CBS 112818	F2RNQ9
218	13691,244	Ribonucleoprotein-associated protein	Trichophyton rubrum CBS 288.86	A0A022W4Q7
219	13707,469	50S ribosomal protein L31e	Nannizzia gypsea CBS 118893	E5QZ74
220	13747,761	hypothetical protein TEQG_03107	Trichophyton equinum CBS 127.97	F2PQA7
221	13779,49	ribosomal protein L31e	Trichophyton equinum CBS 127.97	F2PYV1
222	13812,979	hypothetical protein H109_02490	Trichophyton interdigitale MR816	A0A059JDJ1
223	13828,51	hypothetical protein H109_00904	Trichophyton interdigitale MR816	A0A059JIM0
224	13898,295	hypothetical protein H106_08509	Trichophyton rubrum CBS 735.88	A0A028JAS5
225	13907,214	hypothetical protein H104_01247	Trichophyton rubrum CBS 289.86	A0A022X8H5
226	13921,229	Endoribonuclease L-PSP	Trichophyton equinum CBS 127.97	F2PM80
227	13928,306	hypothetical protein H103_08635	Trichophyton rubrum CBS 288.86	A0A022VNL9
228	13940,37	fatty acid-binding protein	Arthroderma otae CBS 113480	C5FKZ1
229	13959,311	fatty acid-binding protein	Trichophyton equinum CBS 127.97	F2PYU9
230	13992,269	NADH-ubiquinone oxidoreductase subunit GRIM-19	Trichophyton equinum CBS 127.97	F2Q1E2
231	14060,652	hypothetical protein TRV_05526	Trichophyton verrucosum HKI 0517	D4DEF9
232	14064,29	NADH-ubiquinone oxidoreductase subunit GRIM-19	Trichophyton tonsurans CBS 112818	F2SAI5
233	14066,281	hypothetical protein TESG_00081	Trichophyton tonsurans CBS 112818	F2RMF9
234	14100,265	hypothetical protein H109_07116	Trichophyton interdigitale MR816	A0A059J096
235	14139,376	Mitochondrial import membrane translocase subunit TIM16	Trichophyton rubrum MR1448	A0A022YBT0
236	14167,642	Histone H2A	Arthroderma otae CBS 113480	C5G085
237	14170,526	hypothetical protein H101_01637	Trichophyton interdigitale H6	A0A022ULZ8
238	14186,376	hypothetical protein MGYG_00561	Nannizzia gypsea CBS 118893	E5R0G1
239	14227,199	conserved eukaryotic protein	Trichophyton benhamiae CBS 112371	D4AY16
240	14255,194	hypothetical protein MGYG_04653	Nannizzia gypsea CBS 118893	E4UVZ0
241	14255,206	hypothetical protein H101_06980	Trichophyton interdigitale H6	A0A022U679
242	14259,429	Prefoldin subunit 6	Trichophyton tonsurans CBS 112818	F2S5M6
243	14262,673	hypothetical protein MGYG_05361	Nannizzia gypsea CBS 118893	E4UVN8
244	14269,246	hypothetical protein H103_06195	Trichophyton rubrum CBS 288.86	A0A022VWA2
245	14283,237	hypothetical protein TEQG_05212	Trichophyton equinum CBS 127.97	F2PW31
246	14288,505	translation factor SUI1	Trichophyton rubrum CBS 118892	F2SH11
247	14309,606	hypothetical protein H101_01399	Trichophyton interdigitale H6	A0A022UNI8
248	14310,821	Histone H2A	Trichophyton rubrum CBS 202.88	A0A023A4J6
249	14312,801	Histone H2A	Trichophyton interdigitale MR816	A0A059J5J3
250	14313,439	hypothetical protein H101_02029	Trichophyton interdigitale H6	A0A022UKK6

ST1. (Continued)

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
251	14317,731	30S ribosomal protein S26e	Nannizzia gypsea CBS 118893	E5R195
252	14327,481	ubiquinol-cytochrome c reductase complex protein	Trichophyton equinum CBS 127.97	F2PMN2
253	14330,727	40S ribosomal protein S26E	Trichophyton rubrum CBS 202.88	A0A023ARS4
254	14349,514	hypothetical protein H102_01220	Trichophyton rubrum CBS 100081	A0A022VIB2
255	14360,97	hypothetical protein TEQG_02759	Trichophyton equinum CBS 127.97	F2PPA8
256	14371,802	Histone H2A	Nannizzia gypsea CBS 118893	E4UXT4
257	14412,652	60S ribosomal protein L22	Trichophyton tonsurans CBS 112818	F2RPK6
258	14415,053	hypothetical protein H112_05223	Trichophyton rubrum D6	A0A059JYN8
259	14490,219	60S ribosomal protein L35	Trichophyton interdigitale H6	A0A022UFE6
260	14497,681	hypothetical protein ARB_01782	Trichophyton benhamiae CBS 112371	D4B011
261	14498,7	hypothetical protein H110_08801	Trichophyton rubrum MR1448	A0A022Y956
262	14514,133	hypothetical protein H105_05254	Trichophyton soudanense CBS 452.61	A0A022XPI4
263	14726,445	hypothetical protein H113_02302	Trichophyton rubrum MR1459	A0A022ZRJ3
264	14729,826	60S ribosomal protein L23	Trichophyton rubrum CBS 100081	A0A022VG47
265	14743,842	60S ribosomal protein L23	Arthroderma otae CBS 113480	C5FSH1
266	14754,014	40S ribosomal protein S22	Arthroderma otae CBS 113480	C5FBG8
267	14768,029	40S ribosomal protein S22	Trichophyton rubrum CBS 288.86	A0A022WGN2
268	14771,492	hypothetical protein ARB_04644	Trichophyton benhamiae CBS 112371	D4AK43
269	14781,514	hypothetical protein TRV_01858	Trichophyton verrucosum HKI 0517	D4D444
270	14872,152	hypothetical protein H100_04861	Trichophyton rubrum MR850	A0A022TL60
271	14892,873	hypothetical protein TEQG_06170	Trichophyton equinum CBS 127.97	F2PZ65
272	14895,521	hypothetical protein TEQG_04466	Trichophyton equinum CBS 127.97	F2PTU3
273	14896,142	hypothetical protein TESG_05979	Trichophyton tonsurans CBS 112818	F2S554
274	14923,526	hypothetical protein H109_01627	Trichophyton interdigitale MR816	A0A059JGI0
275	14953,068	hypothetical protein TERG_08341	Trichophyton rubrum CBS 118892	F2T0K4
276	14961,119	Histone H2B	Nannizzia gypsea CBS 118893	E4UXT5
277	14965,245	hypothetical protein TRV_03415	Trichophyton verrucosum HKI 0517	D4D8H9
278	14991,13	Histone H2B	Arthroderma otae CBS 113480	C5G086
279	14995,257	60S ribosomal protein L26	Trichophyton tonsurans CBS 112818	F2RWJ5
280	14995,257	60S ribosomal protein	Nannizzia gypsea CBS 118893	E5R351
281	15034,321	60S ribosomal protein L26	Arthroderma otae CBS 113480	C5FKB1
282	15126,746	hypothetical protein TESG_04470	Trichophyton tonsurans CBS 112818	F2S0F0
283	15129,692	hypothetical protein H106_08258	Trichophyton rubrum CBS 735.88	A0A028JB85
284	15133,204	Histone H2B	Trichophyton tonsurans CBS 112818	F2RT32
285	15144,739	hypothetical protein H102_08361	Trichophyton rubrum CBS 100081	A0A022UUA8
286	15145,724	hypothetical protein H105_08388	Trichophyton soudanense CBS 452.61	A0A022XFK1
287	15156,738	hypothetical protein H109_05911	Trichophyton interdigitale MR816	A0A059J341
288	15172,733	conserved hypothetical protein	Trichophyton benhamiae CBS 112371	D4AYC5
289	15206,835	hypothetical protein TRV_03257	Trichophyton verrucosum HKI 0517	D4D822
290	15227,739	conserved hypothetical protein	Trichophyton verrucosum HKI 0517	D4D870
291	15234,593	hypothetical protein H107_02338	Trichophyton rubrum CBS 202.88	A0A023ANT4
292	15322,534	hypothetical protein MGYG_07574	Nannizzia gypsea CBS 118893	E4V3J4
293	15350,565	hypothetical protein H109_02391	Trichophyton interdigitale MR816	A0A059JD05

ST1. (Continued)

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
294	15477,104	hypothetical protein H100_03277	Trichophyton rubrum MR850	A0A022TRS4
295	15491,616	Histone H3	Trichophyton rubrum MR850	A0A022TJC5
296	15631,59	hypothetical protein H110_07654	Trichophyton rubrum MR1448	A0A022YDN5
297	15633,568	60S ribosomal protein L27	Arthroderma otae CBS 113480	C5FDL3
298	15635,548	60S ribosomal protein L27-A	Nannizzia gypsea CBS 118893	E5QZY6
299	15661,601	hypothetical protein H101_02193	Trichophyton interdigitale H6	A0A022UKC2
300	15800,748	40S ribosomal protein S23	Arthroderma otae CBS 113480	C5G0P7
301	15809,909	hypothetical protein H109_03069	Trichophyton interdigitale MR816	A0A059JB33
302	15831,905	hypothetical protein H103_04287	Trichophyton rubrum CBS 288.86	A0A022W3L4
303	15836,759	30S ribosomal protein S26e	Nannizzia gypsea CBS 118893	E4V6M9
304	15839,619	40S ribosomal protein S16	Trichophyton soudanense CBS 452.61	A0A022XH64
305	15844,76	Cu,Zn superoxide dismutase	Trichophyton tonsurans CBS 112818	F2RNE8
306	15850,774	40S ribosomal protein S23	Trichophyton interdigitale MR816	A0A059J9K0
307	15918,812	Superoxide dismutase [Cu-Zn]	Trichophyton interdigitale MR816	A0A059J130
308	15922,408	glycine-rich RNA-binding protein	Trichophyton tonsurans CBS 112818	F2S5E4
309	15922,781	Superoxide dismutase [Cu-Zn]	Trichophyton rubrum CBS 735.88	A0A028JNY9
310	15961,467	hypothetical protein H109_07937	Trichophyton interdigitale MR816	A0A059IX40
311	15974,787	Cu Zn superoxide dismutase	Arthroderma otae CBS 113480	C5FII9
312	16067,827	superoxide dismutase	Nannizzia gypsea CBS 118893	E4UUM7
313	16153,848	60S ribosomal protein L27-A	Trichophyton equinum CBS 127.97	F2PW54
314	16275,254	50S ribosomal protein YmL27	Trichophyton tonsurans CBS 112818	F2S4R8
315	16278,686	40S ribosomal protein S17	Nannizzia gypsea CBS 118893	E5R1Q0
316	16286,727	40S ribosomal protein S17	Arthroderma otae CBS 113480	C5FBZ3
317	16312,198	ssDNA binding protein	Trichophyton tonsurans CBS 112818	F2S609
318	16347,112	Nascent polypeptide-associated complex subunit beta	Trichophyton rubrum CBS 118892	F2SBS6
319	16354,245	hypothetical protein H106_02934	Trichophyton rubrum CBS 735.88	A0A028JSG5
320	16423,484	40S ribosomal protein S19	Trichophyton rubrum CBS 289.86	A0A022X3Y1
321	16427,738	CHCH domain-containing protein	Trichophyton equinum CBS 127.97	F2Q5Z1
322	16447,695	Ribosomal protein L28e	Trichophyton tonsurans CBS 112818	F2RYV1
323	16627,598	hypothetical protein H109_05054	Trichophyton interdigitale MR816	A0A059J5P8
324	16730,119	ribosomal protein L14	Trichophyton tonsurans CBS 112818	F2RPU3
325	16801,893	hypothetical protein H109_01920	Trichophyton interdigitale MR816	A0A059JF24
326	16809,25	40S ribosomal protein S13	Arthroderma otae CBS 113480	C5FT17
327	16832,242	40S ribosomal protein	Trichophyton interdigitale MR816	A0A059JES5
328	16846,258	40S ribosomal protein	Trichophyton soudanense CBS 452.61	A0A022Y0M1
329	16871,584	Nucleoside diphosphate kinase A	Trichophyton equinum CBS 127.97	F2PS09
330	16874,234	60S ribosomal protein L14	Arthroderma otae CBS 113480	C5FKR8
331	16875,486	hypothetical protein H113_04719	Trichophyton rubrum MR1459	A0A022ZHM3
332	16875,965	hypothetical protein H113_02692	Trichophyton rubrum MR1459	A0A022ZRK5
333	16885,6	Nucleoside diphosphate kinase	Trichophyton tonsurans CBS 112818	F2RNW0
334	16916,594	Nucleoside diphosphate kinase	Trichophyton soudanense CBS 452.61	A0A022XRI5
335	16924,057	60S ribosomal protein L27a	Trichophyton interdigitale MR816	A0A059J573
336	16952,063	60S ribosomal protein L28	Trichophyton equinum CBS 127.97	F2PKV4

ST1. (Continued)

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
337	17001,879	Calmodulin	Trichophyton rubrum MR850	A0A022TTW9
338	17281,629	hypothetical protein H109_03367	Trichophyton interdigitale MR816	A0A059JA88
339	**17474,951**	**hypothetical protein TESG_03051**	**Trichophyton tonsurans CBS 112818**	**F2RWA2**
340	17574,482	40S ribosomal protein S15	Nannizzia gypsea CBS 118893	E4UY92
341	17592,703	Eukaryotic translation initiation factor 5A-2	Nannizzia gypsea CBS 118893	E4UQQ1
342	17602,514	40S ribosomal protein S15	Trichophyton rubrum CBS 288.86	A0A022VVB1
343	17604,492	40S ribosomal protein S15	Arthroderma otae CBS 113480	C5FX43
344	17663,789	Woronin body protein HexA, putative	Trichophyton verrucosum HKI 0517	D4DD77
345	17679,797	eukaryotic translation initiation factor 5A	Trichophyton rubrum D6	A0A059K392
346	17716,828	eukaryotic translation initiation factor 5A	Trichophyton interdigitale MR816	A0A059JCQ0
347	17928,84	hypothetical protein H109_04038	Trichophyton interdigitale MR816	A0A059J8S2
348	17939,832	hypothetical protein MGYG_02099	Nannizzia gypsea CBS 118893	E4UPP9
349	18026,865	conserved hypothetical protein	Arthroderma otae CBS 113480	C5FNM6
350	18045,832	hypothetical protein H109_06839	Trichophyton interdigitale MR816	A0A059J028
351	18075,826	60S ribosomal protein L21	Arthroderma otae CBS 113480	C5FZB3
352	18100,213	Nascent polypeptide-associated complex subunit beta	Trichophyton rubrum CBS 118892	F2SBS6
353	18105,836	60S ribosomal protein L21-A	Nannizzia gypsea CBS 118893	E4UW44
354	18342,914	Peptidyl-prolyl cis-trans isomerase, mitochondrial	Trichophyton interdigitale H6	A0A022UFH8
355	18439,168	hypothetical protein H107_08477	Trichophyton rubrum CBS 202.88	A0A023A328
356	18492,008	conserved hypothetical protein	Arthroderma otae CBS 113480	C5FFF0
357	18662,111	hypothetical protein H110_04932	Trichophyton rubrum MR1448	A0A022YLH9
358	18666,869	40S ribosomal protein S17-A	Trichophyton rubrum CBS 100081	A0A022UVW8
359	18668,213	hypothetical protein ARB_06761	Trichophyton benhamiae CBS 112371	D4ARL8
360	18695,355	RecName: Full=E3 ubiquitin ligase complex SCF subunit sconC	Microsporum canis	Q8TGW7
361	18718,15	hypothetical protein TEQG_07210	Trichophyton equinum CBS 127.97	F2Q2B4
362	18730,611	hypothetical protein TESG_03266	Trichophyton tonsurans CBS 112818	F2RWV1
363	18754,344	sulfur metabolite repression control protein C	Arthroderma otae CBS 113480	C5FHU9
364	18759,908	hypothetical protein ARB_05796	Trichophyton benhamiae CBS 112371	D4ANJ1
365	18810,299	E3 ubiquitin ligase complex SCF subunit sconC	Trichophyton rubrum MR1448	A0A022Z3F4
366	18853,348	hypothetical protein H109_00365	Trichophyton interdigitale MR816	A0A059JJL8
367	18877,605	hypothetical protein H103_06914	Trichophyton rubrum CBS 288.86	A0A022VTF3
368	18881,361	translationally controlled tumor protein	Trichophyton equinum CBS 127.97	F2PYM7
369	18906,658	Tropomyosin	Trichophyton equinum CBS 127.97	F2Q2U4
370	18911,346	hypothetical protein H105_00611	Trichophyton soudanense CBS 452.61	A0A022Y6V2
371	19015,793	hypothetical protein H101_06048	Trichophyton interdigitale H6	A0A022U8V1
372	19176,883	woronin body major protein	Trichophyton rubrum CBS 288.86	A0A022W205
373	19424,605	hypothetical protein TEQG_00992	Trichophyton equinum CBS 127.97	F2PJ84
374	19867,73	conserved hypothetical protein	Trichophyton benhamiae CBS 112371	D4B2I1
375	20367,268	hypothetical protein H109_04718	Trichophyton interdigitale MR816	A0A059J7B6

ST1. (Continued)

No.	Mono-isotopic Mass	Description	Taxon	Uniprot entry
376	20380,986	hypothetical protein H100_03179	Trichophyton rubrum MR850	A0A022TS25
377	20399,24	hypothetical protein TEQG_00129	Trichophyton equinum CBS 127.97	F2PGQ7
378	20425,012	60S ribosomal protein L20	Trichophyton interdigitale MR816	A0A059JEI4
379	20531,004	30S ribosomal protein S3	Nannizzia gypsea CBS 118893	E4V353
380	20719,611	hypothetical protein H110_08115	Trichophyton rubrum MR1448	A0A022YBY5
381	20749,473	hypothetical protein H110_06661	Trichophyton rubrum MR1448	A0A022YGH1
382	20763,637	hypothetical protein H101_07261	Trichophyton interdigitale H6	A0A022U5G2
383	20871,463	hypothetical protein H102_05256	Trichophyton rubrum CBS 100081	A0A022V508
384	21068,971	hypothetical protein TRV_06972	Trichophyton verrucosum HKI 0517	D4DIG4
385	21112,191	40S ribosomal protein S15	Trichophyton tonsurans CBS 112818	F2RXI9
386	21121,727	NADH-quinone oxidoreductase	Trichophyton equinum CBS 127.97	F2PI29
387	21389,734	hypothetical protein TEQG_02780	Trichophyton equinum CBS 127.97	F2PPD0
388	21571,766	hypothetical protein H109_01768	Trichophyton interdigitale MR816	A0A059JG04
389	21625,814	cytochrome c subunit vb	Trichophyton tonsurans CBS 112818	F2S9E4
390	21734,984	NAD(P)H:quinone oxidoreductase, type IV	Trichophyton interdigitale MR816	A0A059JGJ7
391	21829,053	minor allergen Alt a 7	Trichophyton rubrum CBS 100081	A0A022VFI1
392	21852,023	flavodoxin	Trichophyton tonsurans CBS 112818	F2S3M0
393	23663,482	Adenine phosphoribosyltransferase	Trichophyton interdigitale MR816	A0A059JGG8
394	23693,492	Adenine phosphoribosyltransferase	Nannizzia gypsea CBS 118893	E4UZW4
395	23709,488	hypothetical protein ARB_04654	Trichophyton benhamiae CBS 112371	D4AK53
396	23709,488	Adenine phosphoribosyltransferase	Trichophyton rubrum CBS 118892	F2SU94
397	24064,018	nitroreductase	Trichophyton equinum CBS 127.97	F2PVJ3
398	24241,076	60S ribosomal protein L1	Arthroderma otae CBS 113480	C5FH50
399	24255,092	60S ribosomal protein L10A	Trichophyton interdigitale H6	A0A022U6Y0
400	25010,602	hypothetical protein H101_06572, partial	Trichophyton interdigitale H6	A0A022U872
401	25247,771	hypothetical protein H103_02885	Trichophyton rubrum CBS 288.86	A0A022W7V0
402	25250,762	hypothetical protein TEQG_06969	Trichophyton equinum CBS 127.97	F2Q1C0
403	25285,496	translation elongation factor 1 subunit Eef1-beta	Trichophyton equinum CBS 127.97	F2PMT1
404	25699,152	hypothetical protein TRV_02696	Trichophyton verrucosum HKI 0517	D4D6H1
405	25700,148	hypothetical protein TESG_07020	Trichophyton tonsurans CBS 112818	F2S7Z2
406	25713,168	hypothetical protein ARB_00424	Trichophyton benhamiae CBS 112371	D4AW59
407	25728,143	hypothetical protein H109_01174	Trichophyton interdigitale MR816	A0A059JHA5
408	25741,164	hypothetical protein H106_01464	Trichophyton rubrum CBS 735.88	A0A028JZX7
409	25769,195	hypothetical protein H103_01699	Trichophyton rubrum CBS 288.86	A0A022WBM3
410	26122,9	ThiJ/PfpI family protein	Trichophyton benhamiae CBS 112371	D4AMF4
411	26136,916	hypothetical protein TESG_07748	Trichophyton tonsurans CBS 112818	F2SA70
412	26200,879	ThiJ/PfpI family protein	Trichophyton verrucosum HKI 0517	D4D9S0
413	29855,912	hypothetical protein H104_02038	Trichophyton rubrum CBS 289.86	A0A022X6J9

ST2. Strain classification by algorithm A2.

rawfiles	prediction 1	prediction 2	prediction 3	prediction 4	CA
CBS 100084_Tr_1	100084_Tr	100084_Tr	100084_Tr	100084_Tr	1
CBS 100084_Tr_2	100084_Tr	100084_Tr	100084_Tr	100084_Tr	
CBS 100084_Tr_3	100084_Tr	100084_Tr	100084_Tr	100084_Tr	
CBS 100238_Tr_1	100238_Tr	100238_Tr	100238_Tr	100238_Tr	1
CBS 100238_Tr_2	100238_Tr	100238_Tr	100238_Tr	100238_Tr	
CBS 100238_Tr_3	100238_Tr	100238_Tr	100238_Tr	100238_Tr	
CBS 115314_Tr_1	115314_Tr	115314_Tr	115314_Tr	115314_Tr	1
CBS 115314_Tr_2	115314_Tr	115314_Tr	115314_Tr	115314_Tr	
CBS 115314_Tr_3	115314_Tr	115314_Tr	115314_Tr	115314_Tr	
CBS 118534_Tv_1	118534_Tv	118534_Tv	118534_Tv	118534_Tv	1
CBS 118534_Tv_2	118534_Tv	118534_Tv	118534_Tv	118534_Tv	
CBS 118534_Tv_3	118534_Tv	118534_Tv	118534_Tv	118534_Tv	
CBS 118892_Tr_1	118892_Tr	118892_Tr	118892_Tr	118892_Tr	1
CBS 118892_Tr_2	118892_Tr	118892_Tr	118892_Tr	118892_Tr	
CBS 118892_Tr_3	118892_Tr	118892_Tr	118892_Tr	118892_Tr	
CBS 119447_Tv_1	119447_Tv	119447_Tv	119447_Tv	119447_Tv	1
CBS 119447_Tv_2	119447_Tv	119447_Tv	119447_Tv	119447_Tv	
CBS 119447_Tv_3	119447_Tv	119447_Tv	119447_Tv	119447_Tv	
CBS 120316_Tv_1	120316_Tv	120316_Tv	120316_Tv	120316_Tv	1
CBS 120316_Tv_2	120316_Tv	120316_Tv	120316_Tv	120316_Tv	
CBS 120316_Tv_3	120316_Tv	120316_Tv	120316_Tv	120316_Tv	
CBS 120318_Tv_1	120318_Tv	120318_Tv	120318_Tv	120318_Tv	1
CBS 120318_Tv_2	120318_Tv	120318_Tv	120318_Tv	120318_Tv	
CBS 120318_Tv_3	120318_Tv	120318_Tv	120318_Tv	120318_Tv	
CBS 120320_Tv_1	120320_Tv	120320_Tv	120320_Tv	120320_Tv	1
CBS 120320_Tv_2	120320_Tv	120320_Tv	120320_Tv	120320_Tv	
CBS 120320_Tv_3	120320_Tv	120320_Tv	120320_Tv	120320_Tv	
CBS 201.88_Tv_1	201.88_Tv	201.88_Tv	201.88_Tv	201.88_Tv	1
CBS 201.88_Tv_2	201.88_Tv	201.88_Tv	201.88_Tv	201.88_Tv	
CBS 201.88_Tv_3	201.88_Tv	201.88_Tv	201.88_Tv	201.88_Tv	
CBS 202.88_Tr_1	202.88_Tr	202.88_Tr	202.88_Tr	202.88_Tr	1
CBS 202.88_Tr_2	202.88_Tr	202.88_Tr	202.88_Tr	202.88_Tr	
CBS 202.88_Tr_3	202.88_Tr	202.88_Tr	202.88_Tr	202.88_Tr	
CBS 288.86_Tr_1	288.86_Tr	288.86_Tr	288.86_Tr	288.86_Tr	1
CBS 288.86_Tr_2	288.86_Tr	288.86_Tr	288.86_Tr	288.86_Tr	
CBS 288.86_Tr_3	288.86_Tr	288.86_Tr	288.86_Tr	288.86_Tr	
CBS 452.61_Tv_1	452.61_Tv	452.61_Tv	452.61_Tv	452.61_Tv	1
CBS 452.61_Tv_2	452.61_Tv	452.61_Tv	452.61_Tv	452.61_Tv	
CBS 452.61_Tv_3	452.61_Tv	452.61_Tv	452.61_Tv	452.61_Tv	
CBS 318.31_Tt_1	318.31_Tt	318.31_Tt	318.31_Tt	318.31_Tt	1
CBS 318.31_Tt_2	318.31_Tt	318.31_Tt	318.31_Tt	318.31_Tt	
CBS 318.31_Tt_3	318.31_Tt	318.31_Tt	318.31_Tt	318.31_Tt	
CBS 285.30_Tt_1	285.30_Tt	285.30_Tt	285.30_Tt	285.30_Tt	1
CBS 285.30_Tt_2	285.30_Tt	285.30_Tt	285.30_Tt	285.30_Tt	
CBS 285.30_Tt_3	285.30_Tt	285.30_Tt	285.30_Tt	285.30_Tt	
CBS 100080_Te_1	100080_Te	100080_Te	100080_Te	100080_Te	1

ST2. (Continued)

rawfiles	prediction 1	prediction 2	prediction 3	prediction 4	CA
CBS 100080_Te_2	100080_Te	100080_Te	100080_Te	100080_Te	
CBS 100080_Te_3	100080_Te	100080_Te	100080_Te	100080_Te	
CBS 856.71_Tt_1	856.71_Tt	856.71_Tt	856.71_Tt	856.71_Tt	1
CBS 856.71_Tt_2	856.71_Tt	856.71_Tt	856.71_Tt	856.71_Tt	
CBS 856.71_Tt_3	856.71_Tt	856.71_Tt	856.71_Tt	856.71_Tt	
CBS 109033_Tt_1	634.82_Te	109033_Tt	109033_Tt	109033_Tt	0,42
CBS 109033_Tt_2	109033_Tt	634.82_Te	634.82_Te	109033_Tt	
CBS 109033_Tt_3	127.97_Tt	127.97_Tt	127.97_Tt	127.97_Tt	
CBS 112186_Tt_1	112186_Tt	112186_Tt	112186_Tt	112186_Tt	0,92
CBS 112186_Tt_2	112186_Tt	112186_Tt	112186_Tt	112186_Tt	
CBS 112186_Tt_3	112186_Tt	112198_Te	112186_Tt	112186_Tt	
CBS 112188_Te_1	270.66_Te	270.66_Te	270.66_Te	270.66_Te	0,67
CBS 112188_Te_2	112188_Te	112188_Te	112188_Te	112188_Te	
CBS 112188_Te_3	112188_Te	112188_Te	112188_Te	112188_Te	
CBS 112193_Te_1	112198_Te	112198_Te	112193_Te	112193_Te	0,75
CBS 112193_Te_2	112193_Te	112193_Te	270.66_Te	112193_Te	
CBS 112193_Te_3	112193_Te	112193_Te	112193_Te	112193_Te	
CBS 112198_Te_1	112198_Te	112198_Te	112198_Te	112198_Te	0,67
CBS 112198_Te_2	112193_Te	112193_Te	112193_Te	270.66_Te	
CBS 112198_Te_3	112198_Te	112198_Te	112198_Te	112198_Te	
CBS 127.97_Tt_1	109033_Tt	127.97_Tt	109033_Tt	109033_Tt	0,42
CBS 127.97_Tt_2	109033_Tt	127.97_Tt	109033_Tt	109033_Tt	
CBS 127.97_Tt_3	127.97_Tt	109033_Tt	127.97_Tt	127.97_Tt	
CBS 270.66_Te_1	112193_Te	270.66_Te	112193_Te	112198_Te	0,42
CBS 270.66_Te_2	112198_Te	112193_Te	112198_Te	112193_Te	
CBS 270.66_Te_3	270.66_Te	270.66_Te	270.66_Te	270.66_Te	
CBS 634.82_Te_1	634.82_Te	634.82_Te	634.82_Te	634.82_Te	0,83
CBS 634.82_Te_2	634.82_Te	109033_Tt	109033_Tt	634.82_Te	
CBS 634.82_Te_3	634.82_Te	634.82_Te	634.82_Te	634.82_Te	

CHAPTER 4

Molecular and phenotypic characterization of *Nannizzia* (*Arthrodermataceae*)

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Submitted for publication

Abstract

Phylogenetic studies of the family *Arthrodermataceae* have revealed seven monophyletic dermatophyte clades representing the genera *Trichophyton*, *Epidermophyton*, *Nannizzia*, *Lophophyton*, *Paraphyton*, *Microsporum* and *Arthroderma*. Members of the genus *Nannizzia* are geo- or zoophiles that occasionally infect humans. With the newly proposed taxonomy, the genus *Nannizzia* comprises eleven species, i.e. *Nannizzia aenigmatica*, *N. corniculata*, *N. duboisii*, *N. fulva*, *N. gypsea*, *N. nana*, *N. incurvata*, *N. persicolor*, *N. praecox*, and two novel species. *Nannizzia polymorpha* sp. nov. was isolated from skin lesion in a patient from French Guiana. For the strain originally described as *Microsporum racemosum* by Borelli in 1965 we proposed *Nannizzia lorica* nom. nov. All species are fully characterized with five sequenced loci (ITS, LSU, *TUB2*, *RP 60S L1* and *TEF3*), combined with morphology of the asexual form and physiological features. A key to the species based on phenotypic and physiological characters is provided.

Keywords

Dermatophytes, *Nannizzia*, morphology, physiology, multilocus analysis

Introduction

Dermatophytes are keratinophilic fungi that affect nail, hair, and skin of humans and warm-blooded animals. Approximately 20–25 % of the global human population is infected with a dermatophyte at least once per lifetime (1). About thirty clinically relevant dermatophyte species are known, but their taxonomy has been controversial because of incongruence of phenotypic and molecular characters (2). In a historical overview of dermatophyte taxonomy (3), four periods were distinguished, based on methods and main criteria used for species discrimination: (1) clinical features only (1840–1895), (2) clinical features combined with phenotypes in culture (1896–1955), (3) mating (1956–1990), and (4) molecular sequencing (1991–today). The highest number of species names (350) was reached around 1950, when novel taxa had been introduced for clinical and morphological variants. A second but much smaller peak of introductions of new names was reached when sexual morphs of these fungi were discovered which were described as separate entities in accordance with current dual nomenclature of fungi. Today, with the introduction of molecular methods, we realize that clinically relevant dermatophytes are phylogenetically more closely related than what was anticipated. As a consequence, the number of recognized species has been greatly reduced; particularly in anthropophilic dermatophytes numerous taxa have been synonymized with pre-existing entities. In 2016 and 2018 comprehensive multilocus phylogenetic studies of *Arthrodermataceae* were published (3, 4) with a phylogeny that in main traits reflected ecological preferences of species in prevalent host animal and environmental habitats. Seven groups were found to be monophyletic and were accepted as genera; amongst these were *Nannizzia* and *Arthroderma*, names that had previously been reserved for sexual states of members of the family. Precise species delimitations using a polyphasic approach is a subsequent step in revising the taxonomy of *Arthrodermataceae*.

The genus *Nannizzia* was introduced by Stockdale (5) with *Nannizzia incurvata* as a type species to accommodate microsporum–like species producing gymnothecia, which had been discovered earlier e.g. by Nannizzi (1927). The sexual state is covered by spirally twisted peridial hyphae composed of ossiform cells, and contains spherical, evanescent asci containing one-celled, lenticular ascospores. The asexual state is characterized by multiseptate, thin- to rather thick-walled, ornamented macroconidia. Through systematic mating experiments on keratinous media, Stockdale (6) produced sexual states in fungi previously known as *Microsporum fulvum* (Uriburu 1909) and *M. gypseum* (Guiard and Gregorakis 1928). In accordance with prevailing nomenclatural rules, these taxa received additional teleomorph names as *Nannizzia fulva* and *N. gypsea*. Most of the species that were classified in the genus *Nannizzia* were described with double nomenclature after finding the heterothallic sexual form. *Nannizzia persicolor* (7) was introduced for *Trichophyton persicolor* (1910). The predominantly sexual species *Nannizzia corniculata* was synonymized as *Arthroderma corniculatum* (8), since the generic name *Arthroderma* preceded *Nannizzia* with more than 100 years. Today, with molecular as the leading classificatory principle, *Nannizzia* and *Arthroderma* are separated as two independent, holomorphic genera: *Arthroderma* covers a large group of ancestral, mainly geophilic species (3) while *Nannizzia* is located between *Trichophyton* and a preponderantly zoophilic genus, *Microsporum*. Several species were found to cluster in the well-demarcated *Nannizzia* group, such as *N. nana* (3), formerly known as *Microsporum nanum* (9), *Nannizzia duboisii* (3), known as *Microsporum duboisii* (10), and *Nannizzia praecox* (3), known as *Microsporum praecox* (11). A recent addition to the genus was the non-sporulating species *Microsporum aenigmaticum* (12), renamed as *Nannizzia aenigmatica* (3).

The abandoned dual nomenclature in fungi has a profound impact on naming of dermatophytes. The genus *Nannizzia* was initially introduced to describe sexual states of known

dermatophytes revealed after mating. Dried type material, usually consisting of tester strains of opposite mating type and the combined product were deposited in herbaria, while living strains were maintained as epitypes in reference collections. Descriptions of the anamorphs were mostly much older, taxa being erected in a time when material of medical strains was only rarely preserved. Those identities are unclear; the historical anamorph species may not have been identical with the deposited sexual species. For practical reasons, de Hoog et al. (3) took the sexual names as nomenclatural reference. The aim of the present revision is to fully characterize all *Nannizzia* species in a modern sense, combining phenotypic features with molecular data. In the course of this study we found two strains in the CBS reference collection that represented undescribed species in *Nannizzia*, i.e. CBS 450.65, deposited as type of *Microsporum racemosum*, and CBS 121947, maintained as *Microsporum amazonicum*. The study offers a comprehensive taxonomic overview of the whole genus with unambiguous identification tools for species recognition in the clinical laboratory.

Materials and methods

Strains analyzed

All analyzed strains were taken from the CBS reference collection (housed at Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands). The total number of strains identified by their ITS sequence and tested for physiology was 56 (Table 1), with a subset of 32 strains selected for multilocus phylogeny. *Nannizzia fulva* and *N. gypsea* were represented with 10 strains each, followed by *N. incurvata* with 9 strains*, N. persicolor* and *N. nana* 7 strains each, *N. praecox* represented by 6 strains and *N. corniculata* with 3 strains. For four species, i.e. *N. aenigmatica*, *N. lorica*, *N. duboisii*, and *N. polymorpha* only a single strain per species was available. Strains were cultured on Sabouraud's Glucose Agar (SGA) plates, using lyophilized, cryo-preserved or fresh mycelial material as inocula. Most of the cultures were incubated for 7 to 14 days at a temperature of 24 °C.

Sequencing

DNA was extracted from cultures grown on SGA plates using MasterPure™ Yeast DNA Purification Kit from Epicentre (Madison, WI, USA). Molecular studies were performed on five gene regions for 32 strains including: ITS, LSU, partial β-tubulin (*TUB2*), translation elongation factor 3 (*TEF3*) and 60S ribosomal protein L10 (*RP 60S L1*) (13). The equipment and PCR conditions used for all five loci were performed as described in (13). The same loci were utilized to infer the phylogeny of the onygenalean families *Arthrodermataceae* (14) and *Ajellomycetaceae* (15). Obtained sequences were manually edited and stored in a Biolomics database maintained at Westerdijk Institute (16). For subsequent phylogenetic and molecular studies, sequences were aligned in five data sets using Mafft version 7 with default settings (17). The identity matrix of BIOEDIT software version 7.2 was used to calculate the percentage of similarity between sequences of the type trains as representatives of the species for all five loci. The concatenated dataset of 32 *Nannizzia* strains with outgroup *Epidermophyton* CBS 230.76 was created using SequenceMatrix software (18) and subjected to phylogenetic analysis using maximum likelihood (RAxML v.8.0.0) employing GTRCAT model and 1,000 bootstrap replicates (19). Bootstrap branch support above 70 % was considered as significant. A dataset

Table 1. Analyzed *Nannizzia* strains.

CBS no.	Strain	ITS	LSU	*TEF3*	*RP60S L1*	*TUB2*
CBS 385.64	*Nannizzia fulva*†	KT155887	KT155232	KT156215	KT156501	MH378957
CBS 599.66	*Nannizzia fulva*	MF926377		MF898415	MF898360	
CBS 243.64	*Nannizzia fulva*†	MF926375	MF893222	MF898412	MF898334	MF898368
CBS 287.55	*Nannizzia fulva*	MF926376	MH378255	MF898413	MF898336	MF898370
CBS 529.71	*Nannizzia fulva*	MF926378	MF893240	MF898414	MF898358	
CBS 147.66	*Nannizzia fulva*	KT155808	KT155144	KT156149	KT156440	KT155496
CBS 146.66	*Nannizzia fulva*	KT155807	KT155143	KT156148	KT156439	KT155495
CBS 130934	*Nannizzia fulva*†	KT155781	KT155112	KT156125	KT156420	KT155470
CBS 130942	*Nannizzia fulva*	KT155784	KT155115	KT156128	KT156423	KT155473
CBS 167.64	*Nannizzia fulva*	AJ000616				
CBS 168.64	*Nannizzia fulva*	MH378229				
CBS 783.73	*Nannizzia fulva*	MH378230				
CBS 784.73	*Nannizzia fulva*	MH378231				
CBS 366.81	*Nannizzia corniculata*	KT155884	KT155228	KT156211	MH378961	KT155553
CBS 364.81	*Nannizzia corniculata*	MF926379	MF893231	MF898416	MF898344	MF898375
CBS 367.81	*Nannizzia corniculata*	MH378232				
CBS 118893	*Nannizzia gypsea*	KT155732	KT155056	KT156082	KT156375	KT155427
CBS 171.64	*Nannizzia gypsea*	KT155814	KT155151	KT156155	KT156446	KT155501
CBS 258.61	*Nannizzia gypsea*	KT155845	KT155184	KT156177	KT156466	KT155522
CBS 100.64	*Nannizzia gypsea*	KT155664	KT154976		KT156308	
CBS 130939	*Nannizzia gypsea*	KT155625	MH378256	KT155990	KT156288	KT155337
CBS 130936	*Nannizzia gypsea*	KT155783	KT155114	KT156127	KT156422	KT155472
CBS 120675	*Nannizzia gypsea*	KT155745	KT155072	KT156097	KT156390	KT155441
CBS 424.66	*Nannizzia gypsea*	MH378233				
CBS 170.64	*Nannizzia gypsea*	MH378234				
CBS 130813	*Nannizzia gypsea*	MH378235				
CBS 134549	*Nannizzia aenigmatica*	MH378236	MH378258	MH378959	MH378964	MH378956
CBS 450.65	*Nannizzia lorica*	KT155905	KT155250	KT156228	KT156513	KT155568
CBS 349.49	*Nannizzia duboisii*	MF926380	MH378259	MH378960	MF898343	MH378954
CBS 161.69	*Nannizzia incurvata**	MH378237				
CBS 311.61	*Nannizzia incurvata**	MH378238				
CBS 174.64	*Nannizzia incurvata*	KT155816	KT155153	KT156156	KT156447	KT155503
CBS 173.64	*Nannizzia incurvata*	KT155815	KT155152			KT155502
CBS 130948	*Nannizzia incurvata*	KT155790	KT155121	KT156133	KT156429	KT155479
CBS 172.64	*Nannizzia incurvata*	MH378239				
CBS 286.63	*Nannizzia incurvata**	MH378240				
CBS 131912	*Nannizzia incurvata**	MH378241				
CBS 548.82	*Nannizzia incurvata*	MH378242				
CBS 128066	*Nannizzia praecox*	KT155772	KT155102	KT156116	KT156411	KT155462
CBS 128067	*Nannizzia praecox*	KT155773	KT155103	KT156117	KT156412	KT155463
CBS 288.55	*Nannizzia praecox*	MH378243	MH378260	MH378958	MH378962	MH378955
CBS 671.89	*Nannizzia praecox*	MH378244				

Table 1. (Continued)

CBS no.	Strain	ITS	LSU	*TEF3*	*RP60S L1*	*TUB2*
CBS 672.89	*Nannizzia praecox*	MH378245				
CBS 673.89	*Nannizzia praecox*	MH378246				
CBS 421.74	*Nannizzia persicolor*	KT155893	KT155238	KT156220	KT156506	KT155560
CBS 871.70	*Nannizzia persicolor*	KT155656	MH378261	KT156013	MH378963	KT155356
CBS 141038	*Nannizzia persicolor*	MH378247				
CBS 422.74	*Nannizzia persicolor*	MH378248				
CBS 468.74	*Nannizzia persicolor*	AJ000615				
CBS 469.74	*Nannizzia persicolor*	AJ000614				
CBS 141034	*Nannizzia persicolor*	MH378249				
CBS 314.54	*Nannizzia nana*	KT155868	KT155212	KT156198	KT156488	KT155543
CBS 632.82	*Nannizzia nana*	KT155952	KT155297	KT156262	KT156544	KT155593
CBS 633.82	*Nannizzia nana*	MH378250				
CBS 321.61	*Nannizzia nana*	MH378251				
CBS 322.61	*Nannizzia nana*	MH378252				
CBS 728.88	*Nannizzia nana*	MH378253				
CBS 569.80	*Nannizzia nana*	DQ860731				
CBS 121947	*Nannizzia polymorpha*	MH378254	KT155077	KT156099	KT156393	KT155444

Legend: †-strains that were not tested for physiology; *-reclassified strains based on ITS phylogeny (data not shown)

of 241 ITS sequences of strains from *Arthrodermataceae* was subjected to a phylogenetic analysis using the same software and parameters as for the concatenated dataset to confirm monophyletic nature of the genus *Nannizzia* within *Arthrodermataceae* and the phylogenetic position and relationship of its species.

Morphology

Phenotypic and growth characteristics of the isolates were studied on SGA and 2 % malt extract agar (MEA, Oxoid, UK) plates incubated for 3 weeks at 27 °C. Cultures were assessed weekly and colony characteristics including morphology, color of mycelia and exuded pigmentation were recorded. Microscopic features were studied using a slide culture method on SGA and MEA agar blocks. Slides were mounted in clear lactic acid and examined using a differential interference contrast microscope (Nikon Eclipse 80i, Nikon, Japan) equipped with Nikon digital sight DS-5M camera. Micrographs and measurements were taken using NIS-Elements imaging software with a minimum of 10 measurements per structure. Photomicrographs were adjusted and assembled in Adobe Photoshop v. CS5.1.

Physiology

All 56 strains were tested for seven physiological parameters. Data were recorded at two time points, i.e. on days 7 and 14. The ability to hydrolyze urea was tested in Christensen's urea broth (CUB). After inoculation, the cultures were incubated at 24 °C in the dark. A color change from orange to pale-pink, red or purple indicated the production of urease. *Trichophyton mentagrophytes* CBS 318.56 was used as positive control (20). Lipase activity was tested on

Tween 80 opacity test medium (TOTM) (21). Strains that formed a halo of crystals around the colony were considered positive. Growth and color change from yellow (acidic) to red (basic) was followed on Dermatophyte test medium (DTM). Milk hydrolysis (pigment production) was tested on Bromocresol purple-milk solids-glucose agar (BCP-MS-G) according to Fisher & Kane (22). Positive strains change the color of the medium due to casein hydroxylation, resulting in a dark purple-blue color of the agar (23). Beta-hemolysis was tested on sheep blood agar plates (BioMerieux, France). Plates were incubated at 37 °C and positive response was recorded as a transparent zone of clearing. Tolerance to cycloheximide and sodium chloride (NaCl) was tested on SGA plates supplemented with 0.2 % cycloheximide and 3 % and 5 % NaCl. Cardinal temperatures and growth rates were determined on MEA plates at 6–36 °C with 3 °C intervals, and at 37 and 40 °C, measured at day 7 and day 14 of incubation.

Results

Phylogenetic and molecular analyses

An ML phylogenetic study on the ITS dataset confirmed the topology found by de Hoog et al. (3). All species included in the current study clustered in a group defined by *Nannizzia incurvata* as type species, with a bootstrap support of 83 % (Fig. 1). A five-locus alignment matrix was generated for 32 *Nannizzia* strains covering ITS (591 bp), and partial LSU (810 bp), *TUB2* (448 bp), *TEF3* (266 bp) and *RP 60S* L1 (461 bp). GenBank accession numbers of all sequences are given in Table 1, and the resulting ML phylogenetic tree is presented in Fig. 2. The topologies of bootstrap-supported branches of the trees are highly corresponding. Strain CBS 134549 of *N. aenigmatica* grouped with CBS 450.65 (named below as *N. lorica*) at some distance of the core group

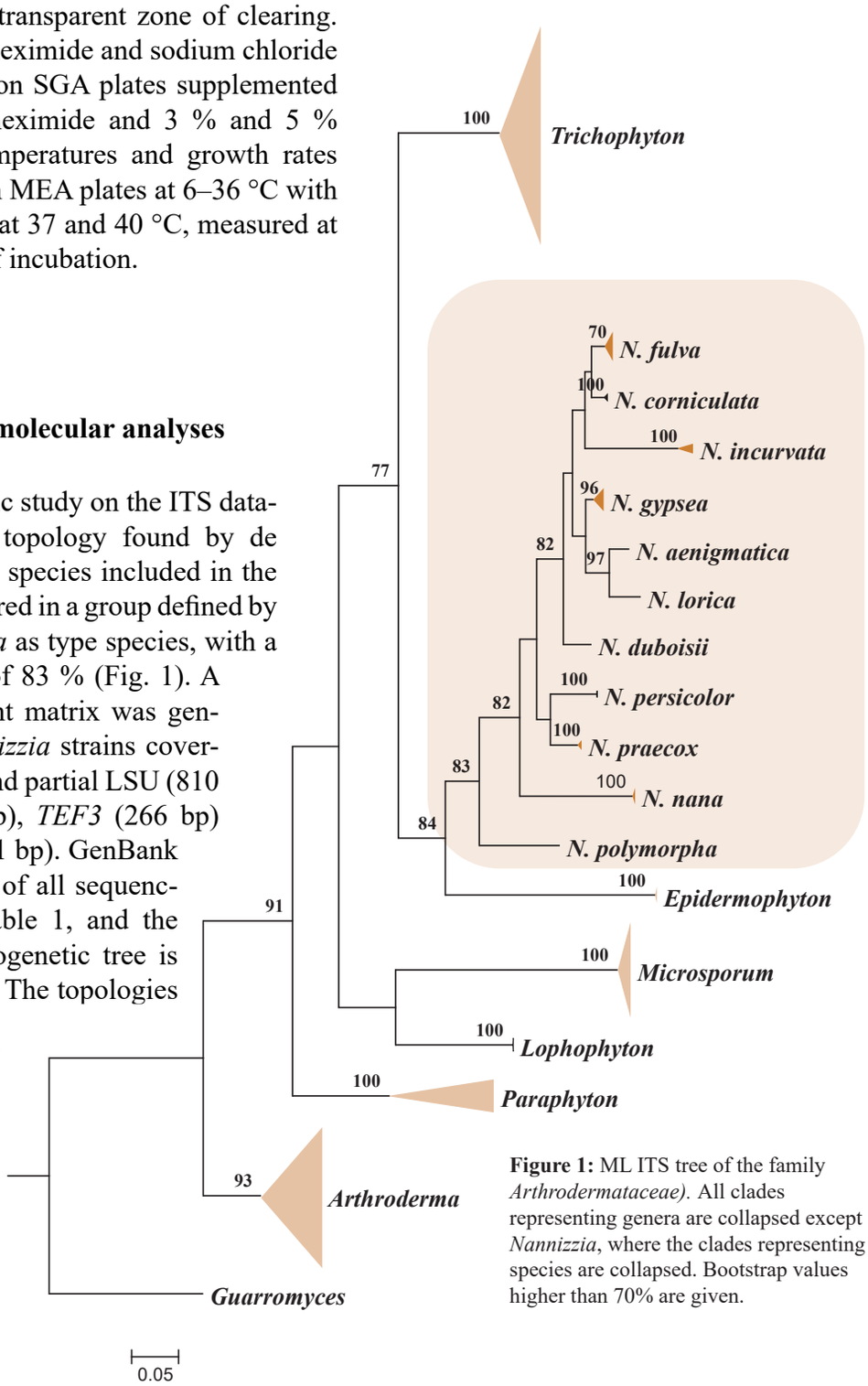

Figure 1: ML ITS tree of the family *Arthrodermataceae).* All clades representing genera are collapsed except *Nannizzia*, where the clades representing species are collapsed. Bootstrap values higher than 70% are given.

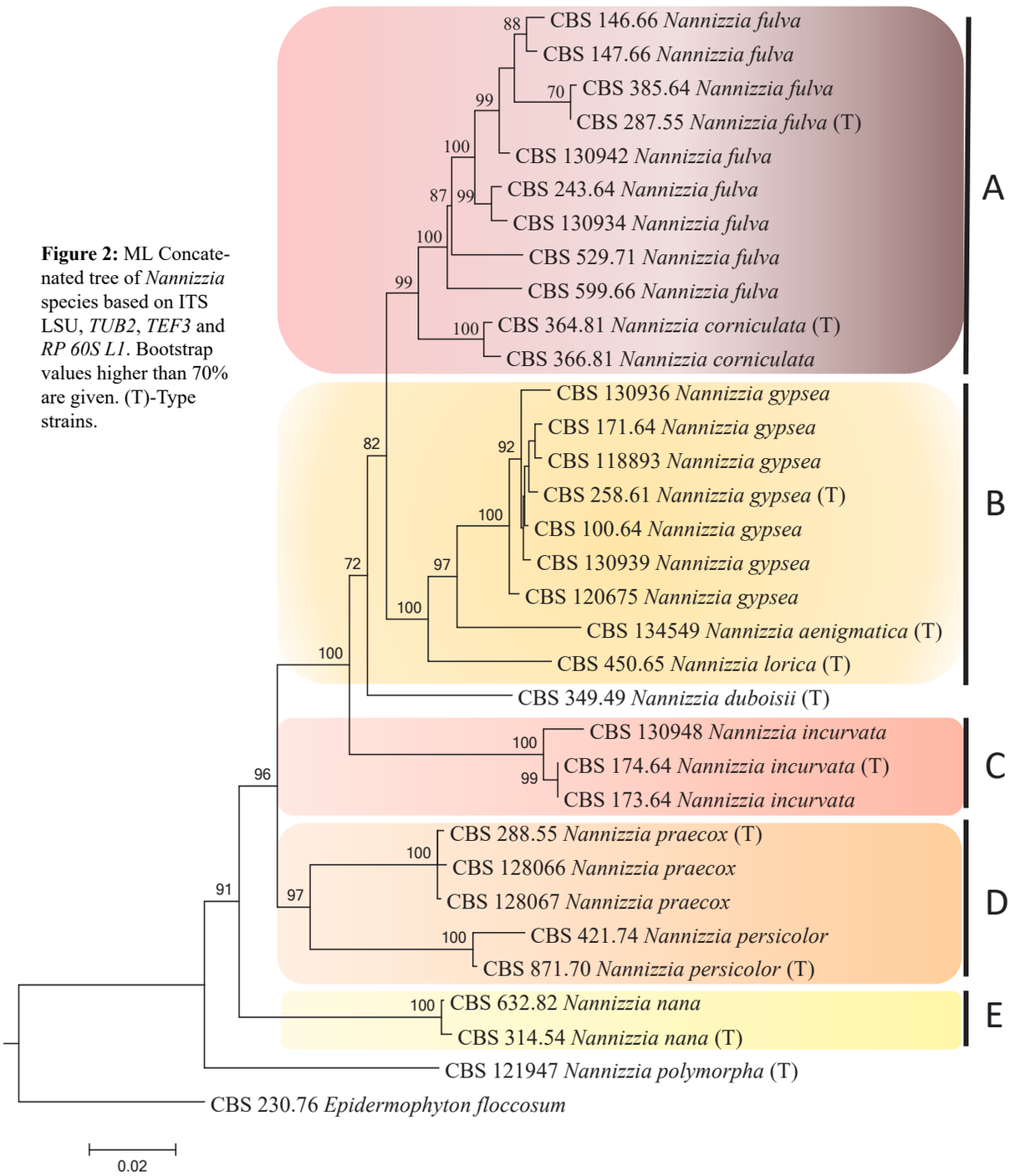

Figure 2: ML Concatenated tree of *Nannizzia* species based on ITS LSU, *TUB2*, *TEF3* and *RP 60S L1*. Bootstrap values higher than 70% are given. (T)-Type strains.

(Clade B) with 100 % BS support and containing *N. gypsea.* Clade A (BS 99 %) is sister to Clade B and comprised *N. fulva* and *N. corniculata*. *Nannizzia duboisii* and *N. incurvata* (Clade C, BS 100%) are paraphyletic to Clades A and B, but cluster with them in 100 % BS supported node. *Nannizzia praecox* and *N. persicolor,* each forming their own subclades with BS of 100 %, formed a separate clade (Clade D, BS 97 %), while *Nannizzia nana* (Clade E, BS 100 %) and *N. polymorpha* took an ancestral position to all species. All branches between clades had BS support values higher than 70 %, except for the lowest branch connecting *N. polymorpha* to remaining species; the position of this single strain is uncertain and may be due to the long

branch attraction. The BS values of all clades representing species with more than 2 strains (seven species) are 100 %.

Table 2. Summarized pairwise comparisons of eleven *Nannizzia* type strains.

Groups of pairwise comparisons	≤90 %	91–95 %	≥96 %
ITS (591 bp)	31	23	1
LSU (810 bp)	0	0	55
TUB2 (448 bp)	37	18	0
RP60S L1 (461 bp)	38	17	0
TEF3 (266 bp)	0	31	24

The identity matrices of the ITS, LSU, *TUB2*, *RP 60S L1* and *TEF3* sequences of type strains are given in Supplementary Table 1. Fifty-five pairwise comparisons between three intervals (≥ 96 %, 91–95 % and ≤ 90 %) are summarized in Table 2. Low sequence similarities were recorded for *BTUB2* and *RP 60S L1*, i.e. 37 and 38 in interval ≤ 90%, 18 and 17 in interval 91–95 %, respectively. No similarity ≥ 96 % was found with these loci. In ITS, 31 pairwise comparisons were ≤ 90%, 23 were in the interval 91–95 %, and one in ≥ 96 %. *TEF3* had no comparisons in the group of ≤ 90 %, 31 were in the interval 91–95 %, and 24 in the group ≥ 96 %. With the LSU locus, all species showed very high sequence similarities, with 100 % identity in 13 comparisons.

Physiology

Results of physiological tests are given in Table 3. All strains grew on Dermatophyte Test Medium, changing the color from yellow to red. In addition, all strains showed growth when cultured on SGA supplemented with 0.2 % cycloheximide, or with 3 % and 5 % NaCl. Contrary to these uniform results, remaining physiological tests (urease production in CUB, lipase activity on TOTM, Milk hydrolysis on BCP-MS-G, ß-hemolysis on blood agar, and temperature

Table 3. Physiological test results of 56 *Nannizzia* strains.

Test	N.fu	N.co	N.gy	N.ae	N.lo	N.du	N.in	N.pr	N.pe	N.na	N.po
	(10)	(3)	(10)	(1)	(1)	(1)	(9)	(6)	(7)	(7)	(1)
DTM	10p	3p	10p	p	p	p	9p	6p	7p	7p	p
Cycloheximide	10p	3p	10p	p	p	p	9p	6p	7p	7p	p
5% NaCl	10p	3p	10p	p	p	p	9p	6p	7p	7p	p
Urease production (CUB)	1n,3p, 6w/p	1n, 2n/p	4p, 6w/p	p	p	w/p	8p, 1n/p	6p	4p, 3w/p	6p,1w/p	p
Lipase activity (TOTM)	10p	3p	7p, 2n/ p,1n	p	n	n/p	9p	6n	4p,3n/p	5p,2n/w	n/p
Milk hydrolysis (BCP-MS-G)	7n,3p	3n	5n,3p, 2n/p	n/w	n	n	8p,1n/p	6n	4n,3w	1n,1p, 5n/p	p
ßeta hemolysis on blood agar	10n	3n	10n	p	n	n/p	9n	6n	7n	1n, 6n/p	n
Growth at 37 °C	6n,4p*	3p	8p,2n/*	n	p	n/*	9p	6n	7p	6p,1n/*	p

Legend ( ), number of analysed strains per species; p, positive; n negative; w, weak; *, poor growth; /, different results recorded at day 7 and day 14.

relations) yielded intraspecific variation in species where more than one strain was available for study. All strains, except *N. fulva* CBS 287.55 and *N. corniculata* CBS 364.81 were urease positive (purple color) after two weeks of incubation. Different results at day 7 and day 14 were recorded for all species represented by more strains except for *N. praecox*, where all six strains were positive in one week. All strains of *N. fulva*, *N. corniculata* and *N. incurvata* were positive for lipase forming clear halos around colonies, but strains of *N. praecox* were negative. In most of the cases, however, no clear halo was visible, but crystals could be observed in the agar under the colony. Nine out of 10 *N. gypsea* strains were positive after 14 days, while one (CBS 120675) remained negative at both time points. Three strains of *N. persicolor* showed negative reaction after 7 days, but all were positive at day 14 of growth. Five *N. nana* strains were positive and two showed negative to weak reactions on day 7 and day 14, respectively. Milk hydrolysis test was negative for all *N. corniculata* strains and for all *N. praecox* strains. For all other species this test proved to be highly variable. The color change on BCP-MS-G medium was not obvious as most species exuded pigments into the medium which interfered with reading. ß-hemolysis was not observed in all tested species except for six strains of *N. nana*, the one of *N. aenigmatica* and *N. duboisii*. The positive *N. nana* strains showed βeta hemolysis only after 14 days; one strain remained negative. *N. duboisii* CBS was also positive only after 14 days while *N. aenigmatica* was positive at both recording points. Growth at 37 °C was observed in *N. corniculata*, *N incurvata*, *N. persicolor, N. lorica* and *N. polymorpha*, while 6 strains of *N. fulva* and the strains of *N. praecox* and *N. aenigmatica* showed no growth. *N. duboisii*, two strains of *N. gypsea* and one strain of *N. nana* showed no growth at day 7 and poor growth at day 14.

Morphology

The genus *Nannizzia* is characterized by expanding cottony to powdery colonies which can be creamy whitish, yellow, orange, brown or reddish. The colony reverse usually shows bright yellow, orange, or reddish brown pigmentation. The asexual form has abundant, 1–8-septate, thin- or moderately thick- and rough-walled macroconidia which are cylindrical, cigar-shaped, ellipsoidal or fusiform, although in *N. aenigmatica* and *N. lorica* sporulation is not known. Microconidia are aseptate or rarely with one septa, arranged individually or in small clusters, sessile or short-stalked, (sub)spherical, or clavate, usually abundant, but scant in *N. aenigmatica* and *N. praecox*. The fungi are heterothallic; ascomata are gymnothecia which are morphologically highly monomorphic in *Arthrodermataceae*.

Diagnosis

In clinical practice the genus *Nannizzia* differs from *Lophophyton, Microsporum* and *Paraphyton* by relatively thin-walled macroconidia, and *Lophophyton* by maximally 8- against up to 11-septate macroconidia. *Trichophyton* differs by having scant, thin- and smooth-walled macroconidia. Some species of *Arthroderma* are similar to, but differ from *Nannizzia* by larger, thick-walled macroconidia. *Epidermophyton* is easily distinguished by absence of microconidia and smooth-walled macroconidia.

Key to Nannizzia species

1a.	Macroconidia present …….....	2
1b.	Macroconidia absent …….....	10
2a.	Microconidia abundant …….....	3
2b.	Microconidia scant or absent; macroconidia abundant, 3–8-septate, borne on branched conidiophores; lipase and milk hydrolysis negative; no growth at 37°C …….....	*N. praecox*
3a.	Macroconida short obovoidal to clavate, 1- or rarely 2-septated.....	*N. nana*
3b.	Macroconida with more than 2 septa …….....	4
4a.	Macroconidia polymorphic: cylindrical to clavate with rounded apex, 1–4-septate, or ovoidal to obpyriform with or without septa ….....….	*N. polymorpha*
4b.	Macroconidia homogeneous in shape .....….....……	5
5a.	Macroconidia mostly with less than 6 septa, broadly ellipsoidal to fusiform; colonies yellowish-buff or tan, granular…….....….	*N. gypsea*
5b.	Macroconidia often with 6, 7 or 8 septa…….....…..	6
6a	β-hemolysis positive; BCP-MS-G negative; lipase activity slow; poor growth at 37 °C; colonies creamy white, woolly or powdery with yellow reverse; macroconidia 2–6-septate.....	*N. duboisii*
6b.	β hemolysis negative; remaining characters not combined …….....….	7
7a.	Macroconidia with less than 7 septa, strictly fusiform; colonies finely powdery, pale brown to buff; BCP-MS-G positive …….....….	*N. incurvata*
7b.	Macroconidia often with 7, up to 8 septa …….....……	8
8a	Macroconidia echinulate, with up to 8 septa; colonies pale yellow buff with yellow reverse; lipase positive; BCP-MS-G negative…….....……	*N. corniculata*
8b	Macroconidia verruculose or with warty projection, 3–7-septate, cylindrical to elongate fusiform or cigar-shaped; colony reverse reddish brown.…….....……	9
9a	Microconidia abundant; macroconidia cigar-shaped or narrow cylindrical to elongate fusiform with rounded or pointed apex; growth at 37 °C ….....	*N. persicolor*
9b	Microconidia scant; macroconidia elongate fusiform with pointed apex; lipase positive……....	*N. fulva*
10a.	Microconidia abundant; colonies white to pale buff with reddish-brown reverse; growth at 37 °C .	*N. lorica*
10b.	Microconidia absent or scant; colonies ochraceous with yellow-orange reverse; no growth at 37 °C..	*N. aenigmatica*

Taxonomy

Nannizzia aenigmatica (Hubka, Dobiášová & Kolařík) Gräser & de Hoog — MycoBank MB824533; Fig. 3

Type. CZECH REPUBLIC, Ostrava, skin lesion on the wrist of a woman, S. Dobiášová; culture ex-type CCF 4608 = CBS 134549.

Colonies on SGA circular, flat, radially or irregularly furrowed, pale ochraceous with some brownish sectors, slightly zonate with dark yellow margin consisting of submerged mycelium, reverse dark yellow-orange. Colonies on MEA circular with cottony aerial mycelia, creamy white or pale ochraceous, reverse yellow-orange in the center becoming faint towards the margin. Hyphae septate, hyaline to pale yellow, smooth-walled, sometimes curved and intertwined forming dense masses, racquet hyphae present. Macroconidia not observed. Microconidia rare, formed on undifferentiated hyphae, ovoidal or clavate. Chlamydospore-like structures abundant.

Urease positive, lipase positive, milk hydrolysis negative to weakly positive, ß-hemolysis positive, no growth at 37 °C.

Nannizzia polymorpha Dukik, S.A. Ahmed & de Hoog, **sp. nov.** — MycoBank MB825465, Fig. 4

Etymology: The species epithet refers to the morphologically variable conidia that characterize the species.

Type. FRENCH GUYANA, human skin lesion, N. Contet-Audonneau, Holotype CBS-H-23607, culture ex-type CBS 121947.

Colonies on SGA circular, yellow buff, powdery or lanose with fluffy tuft aerial mycelia, reverse yellow. Colonies on MEA creamy white, radiating, granular, cottony, reverse dark yellow. Hyphae hyaline, septate, spiral hyphae present. Macroconidia thin- or moderately thick-walled, smooth-walled or slightly verrucose, borne on branched or unbranched conidiophores while some are sessile, arrange individually or in clusters. Two types of macoconidia were present: abundant type, 16–59 × 7–17 µm, with 1–4 or rarely 5 septa, cylindrical or clavate with blunt rounded or slightly pointed apex, mostly on clusters. Rare type, 23–43 × 15–29 µm, 0–1-septate, ovate egg-shaped or obpyriform with a tapering pointed or slightly rounded apex. Microconidia 3–10 × 7–15µm, sessile or short-stalked, 0–1-septate, arranged mostly in small clusters but also alongside undifferentiated hyphae. Chlamydospore-like structure present. Optimum growth temperature 30 °C, colonies reach 68 mm diameter after 2 weeks, grow at 37 °C.
Urease positive, lipase positive after two weeks, milk hydrolysis positive, ß-hemolysis negative.

Nannizzia corniculata Takashio & De Vroey, — MycoBank MB110836; Fig. 5

Type. SOMALIA, Las Anod, soil, 1966, Ch. De Vroey & M. Takashio, culture ex-type CBS 364.81 = ATCC 46541 = IHEM 4409 = RV 20845.

Colonies on SGA pale yellow buff, circular, woolly and downy, reverse yellow. Colonies on MEA circular, creamy white powdery, reverse yellow orange. Macroconidia 1–8-septate, straight or slightly curved, thin- or moderately thick-walled, echinulate, cigar-shaped or narrow cylindrical with pointed or round apex, borne on unbranched or branched conidiophores. Microconidia sessile or short-stalked, single-celled, clavate.
Urease variable, lipase positive, milk hydrolysis negative, ß-hemolysis negative, grow at 37 °C.

Nannizzia duboisii (Vanbreuseghem) Gräser & de Hoog, — MycoBank MB824534; Fig. 6

Type. ZAIRE, Kangu, skin of infant, R. Vanbreuseghem, culture ex-type CBS 349.49.

Colonies on SGA circular, creamy white, velvety to woolly or powdery, reverse yellowish.

Colonies on MEA creamy white, velvety with thin, cottony margin, reverse yellow orange. Macroconidia 2–6-septate, rough-walled, thin- or moderately thick-walled, fusiform with pointed apex. Microconidia sessile alongside undifferentiated hyphae or stalked, clavate to obovoidal, sometimes present in small clusters. Chlamydospore like structures present.
Urease weak to positive, lipase positive after two weeks, milk hydrolysis negative, ß-hemolysis positive after two weeks, poor growth at 37 °C.

Nannizzia fulva (Uriburu) Stockdale — MycoBank MB335065; Fig. 7

Type. ARGENTINA, from human, E. Rivalier, culture ex-type CBS 287.55.

Colonies on SGA beige-buff to pink buff, finely or coarsely granular, farinose, circular or irregularly radiating, flat or heaped and convoluted at the center causing cracks in the agar, reverse buff-orange to reddish brown at the center. Colonies on MEA beige-buff, powdery at the center with a white cottony periphery, circular, reverse buff-orange or red. Macroconidia 3–7-septate, thin- or moderately thick-walled, verruculose, usually elongated fusiform or lanceolate to cylindrical and slightly tapering towards each end, borne on short, unbranched or branched conidiophores, some strains produce coherent non-maturating elongated conidia. Microconidia scant, sessile or short-stalked, 0–1-septate, obovoidal or clavate, borne on cylindrical or slightly swollen mycelia. Spiral hyphae present.
Urease and milk hydrolysis variable, lipase positive, ß-hemolysis negative, grow at 37 °C negative to poor.

Nannizzia gypsea (Nannizzi) Stockdale — MycoBank MB33506; Fig. 8

Type. AUSTRALIA, New South Wales, Turramura, soil, 1960, D.M. Griffin, culture ex-type CBS 258.61 = CBS 169.64 = IMI 80558.

Colonies on SDA radiating, deep cream, yellowish-buff or tan, coarsely granular or powdery, reverse yellowish-buff with some pinkish tinges. Colonies on MEA dark cream to yellowish or pale-buff, radiating, granular, some cultures form white cottony or fluffy tufts of aerial mycelium, reverse buff with yellowish-brown or reddish-brown pigmented spots. Macroconidia 2–5-septate, symmetrical, broadly ellipsoidal to fusiform, tapering toward the ends and slightly rounded at the apex, thin-walled, verrucose, borne individually on short branches alongside hyphae or in clusters. Microconidia sessile or stalked alongside undifferentiated hyphae, obovoidal or clavate, 0–1-septate.
Urease weak to positive, lipase and milk hydrolysis variable, ß-hemolysis negative, grow at 37 °C positive with the exception of two strains with none to poor growth.

Nannizzia incurvata Stockdale — MycoBank MB335068; Fig. 9

Type. UNITED KINGDOM, human skin, P.M. Stockdale, culture ex-type CBS 174.64 = IMI 82777 = NCPF 236.

Colonies on SGA pale brown to buff, powdery or finely granular with some white tuft

cottony mycelia at the center, reverse orange or reddish yellow or cinnamon. Colonies on MEA circular, creamy to pale buff, powdery with some white tuft cottony mycelia, reverse reddish yellow or cinnamon. Macroconidia 1–6-septate, thin- or moderately thick-walled, verrucose, fusiform, formed on unbranched or repeatedly branched conidiophores. Microconidia sub-spherical or clavate, sessile alongside undifferentiated hyphae.
Urease and milk hydrolysis mostly positive, lipase positive, ß-hemolysis negative, grow at 37 °C.

Nannizzia nana (Fuentes) Gräser & de Hoog — MycoBank MB554303; Fig. 10

Type. Country UNKNOWN, kerion of human scalp, C.A. Fuentes, culture ex-type CBS 314.54 = ATCC 11832.

Colonies on SGA expanding and radiating, creamy white or light buff, cottony or powdery, reverse dark brown to reddish-brown at the center. Colonies on MEA white pale yellow or buff, powdery to velvety at the center and white cottony towards the margin, reverse orange. Macroconidia 1- or rarely 2-septate and somtimes aseptate, thin-walled, verrucose with some warty projections, obovoidal to clavate, with truncate bases. Microconidia sessile, ovoidal or clavate.
Urease mostly positive, lipase positive or weak, milk hydrolysis variable, ß-hemolysis mostly positive after two weeks, grow at 37 °C with the exception of one strain with none to poor growth.

Nannizzia persicolor (Padhye, Ajello & McGinnis) Gräser & de Hoog — MycoBank MB33507; Fig. 11

Type. BULGARIA, human skin lesion, V.A. Balabanov, culture ex-type CBS 871.70.

Colonies on SGA expanding pale yellow, circular, flat, velvety or fluffy powdery, reverse brownish red or orange red at the center becoming faint towards the margin. Colonies on MEA granular, pale yellowish-buff at the center with cottony, white margin, reverse ochraceous. Macroconidia 3–7-septate, thin- and rough-walled with scattered warty projections, elongated fusiform to cigar-shaped. Microconidia ovoidal, (sub)spherical, sessile, mostly in clusters. Spiral hyphae present.
Urease mostly positive or weak, lipase positive (some strains turned positive only after two weeks), milk hydrolysis on BCP-MS-G negative or weak, ß-hemolysis negative, grow at 37 °C.

Nannizzia praecox (Rivalier ex A.A. Padhye, Ajello & McGinnis) Gräser & de Hoog) — MycoBank MB 629706; Fig. 12

Type. FRANCE, skin lesion on the wrist of a man, Rivalier, culture ex-type CDC B-4819D= ATCC 66852, authentic strain CBS 288.55, human, Rivalier.

Colonies on SGA creamy to yellowish-tan or buff, radiating, granular or powdery with suede-like cloudy growth waves, reverse yellow-orange. On MEA buff, powdery to finely granular with cottony white tuft aerial mycelia, reverse yellow-orange. Macroconidia abundant,

3–8-septate, thin- and rough-walled, long ellipsoidal or cigar-shaped or sometimes clavate, often borne on complex branched conidiophores. Microconidia rarely present, thin-walled, sessile, 0–1-septate, pyriform or clavate. Chlamydospore-like structures present.
There was no intraspecific variability in this species; urease positive, lipase negative, milk hydrolysis negative, ß-hemolysis negative, no growth at 37 °C.

Nannizzia lorica (Rush-Munro, J.M.B. Smith & Borelli) Dukik, S.A. Ahmed & de Hoog, ***nom. nov.*** — MycoBank MB825523; Fig. 13

Etymology: The species epithet refers to the source, mammal fur, from which the type strain was recovered (*Rattus rattus*).

Type. VENEZUELA, hair of *Rattus rattus*, D. Borelli, Holotype Herbarium needed, culture ex-type CBS 450.65 (type of *Microsporum racemosum*).

Colonies on SGA white to pale buff, radiating, velvety or powdery, reverse reddish-brown. On MEA creamy white, cottony to finely granular, reverse faint yellow. Macroconidia not observed when the type strain was examined. In the original description by Borelli ((24) macroconidia was reported "1–5 rarely 6-septa, thin- or moderately thick-walled, echinulate, fusiform, 55–65 × 12–15 µm". Microconidia abundant, hyaline, in various sizes and shapes but mostly ovoidal to clavate, born single or in grape-like clusters. Optimum temperature 27 °C, colony reaching 70 mm diameter after 2 weeks.
Urease positive, lipase negative, milk hydrolysis negative, ß-hemolysis negative, grow at 37 °C.

Discussion

The molecular phylogeny of the family *Arthrodermataceae* corresponds in main traits with the classical ecological grouping of dermatophytes into geo-, zoo- and anthropophilic species. Anthropophilic species are restricted to the genera *Trichophyton* and *Epidermophyton*, while geophilic species are mainly found in the genera *Arthroderma* and *Nannizzia*. Zoophilic dermatophytes, i.e. species prevalently found in the fur of a particular (group of) mammal host species, is difficult to define, as also geophilic species can be carried asymptomatically by burrowing animals. Several members of the genus *Nannizzia* have been associated with particular mammal hosts, although some are found in soil or causing infections in humans, hence they are considered as opportunistic pathogens. The overall estimated prevalence of human infections caused by species of the *N. gypsea* complex (*N. gypsea*, *N. fulva* and *N. incurvata*) in Europe is around 1 % of all dermatophytosis (25). In humans they mainly cause tinea corporis (26-28). A specific clinical feature seems to be a white, paint-like hypergrowth on the scrotum (29-31). Cases in immunocompromised patients such as with HIV or Lupus tend to be severe (32, 33).

Nannizzia persicolor was isolated from European bank and field voles (English 1966) and it is occasionally causing tinea corporis, tinea capitis or tinea pedis in humans (34-37) Although, Muller et al. (38) reported 16 cases of dermatophytosis in dogs due to this species, no human infection acquired via contact with symptomatic or asymptomatic domestic animals was noted. In India, *N. persicolor* is the prevalent species causing human infections (39). In contrast, *Nannizzia praecox* has been described as rare cause of human skin lesions (40),

causing tinea corporis and tinea capitis (41). The infections are reported to be acquired from the equine environment (40, 42). *Nannizzia corniculata* is a geophilic and has not been encountered as a pathogen (43). *Nannizzia lorica* was first isolated from an asymptomatic rat (24) and later from soil, but no proven human cases have been reported (44). A strain molecularly identified as closest to *N. lorica* was recovered from onychomycosis (45). *Nannizzia nana* was originally reported from pigs causing chronic circular lesions on pigs ears (46, 47). Human infections in the form of tinea capitis and tinea corporis occur through direct contact with the infected animals (48-50). All seven strains analyzed in our study originated from humans but the human host certainly has a sampling bias. The remaining species which all are represented by a single strain, i.e. *N. duboisii*, *N. aenigmatica*, *N. lorica*, and *N. polymorpha*, were all derived from human infections.

The era of molecular taxonomy and phylogeny for this group of fungi started in the early 1990s, with an analysis of restriction patterns of mitochondrial DNA (51). The study revealed that *Nannizzia* species had distinct restriction patterns and this matched with subsequent ITS (2) and multilocus studies (3). Later phylogenetic studies (52, 53) all demonstrated highly concordant topologies of trees, being largely insensitive to strain and taxon sampling effects. The last described species in *Nannizzia*, *M. aenigmaticum*, was published separately as an etiologic agent of tinea corporis (12). The authors recognized eight species in *Microsporum gypseum* complex based on phylogeny of two loci, ITS and *β*-tubulin. Their analysis placed the new species closest to *Microsporum gypseum* and *M. fulvum*, which now are *Nannizzia* species. In our analyses *M. aenigmaticum* is part of the *Nannizzia gypseum* group, as nearest neighbor of *N. lorica*.

Our taxonomic study of *Nannizzia* supplemented earlier ITS data presented by de Hoog et al. (3) and Zhan et al. (4). Isolate CBS 121947 proved to represent a hitherto undescribed *Nannizzia* species. *Microsporum racemosum* CBS 450.65 was described by Borelli in 1965 (24) from the fur of a rat in Venezuela. Borelli was coauthor of a paper describing the supposed teleomorph obtained after crossing of two strains from soil in Georgia, U.S.A. and which were deposited as isotypes of *Nannizzia racemosa* (Rush-Munro et al. 1970). The strains were however re-identified as *Paraphyton cookei* (syn. *Microsporum cookei*), a name preceding *N. racemosa* by two decades (Ajello 1959). The strain CBS 450.56 is the type of the asexual species *Microsporum racemosum* which clusters in *Nannizzia* rather than in *Paraphyton* and which thus should be maintained as a separate species. However, since *Nannizzia racemosa* already exists as a synonym of *P. cookei*, a new name, *N. lorica*, is introduced here. Another strain of *M. racemosum* causing an onychomycosis in a Spanish female was reported (45). In this publication, the authors stated that the comparison of its ITS sequence identified the fungus as *M. racemosum* strain of Borelli, CBS 450.65. However, the GenBank accession number and CBS or other reference collection code of this strain were not published, thus it could not be included in our analysis. Strain CBS 121947 from a facial lesion of a patient in French Guiana was morphologically identified as *Microsporum amazonicum*, but combined molecular and phenotypic features supported its identity as a novel species, described in this paper as *Nannizzia polymorpha*.

Hubka et al. (12) calculated degrees of sequence similarity between species for ITS and *β*-tubulin; the found values for these loci are comparable to the ones obtained in our study. In general, for all loci except LSU the distances between species are significantly larger than those between *Trichophyton* species. In assessment of the phylogenetic power of five loci (4) ITS was shown to be the best locus for species identification in *Arthrodermataceae*, although precise

borderlines between anthropophilic species still have to be determined.

Morphological and physiological characters have been evaluated in *Nannizzia* to assess their discriminative value (23, 54, 55). Teleomorph data are not useful for diagnostics, since species are heterothallic and the sexual states are highly similar between species. This is a general feature in *Onygenales*, where e.g. sexual states of *Emmonsia, Blastomyces* and *Histoplasma* all have been attributed to *Ajellomyces* (56). Some *Nannizzia* species can easily be distinguished by their growth characteristics and the shape of their macroconidia. In general, *Nannizia* species produce thin- or moderately thick- and rough-walled macroconida with 2–7 cells. However, *N. lorica* and *N. aenigmatica* lacked macroconidial sporulation. The species also produce abundant 1- or 2-celled, subspherical or ovoidal microconidia, except in *N. praecox* and *N. aenigmatica* where they were absent or scant. We combined morphological features with physiological activity of the strains to support species distinction and enhance routine identification. Despite elaborate morphology of most species, molecular identification remains indispensable since phenotypic characters are variable in all species treated.

Nannizzia species grow on SGA plates supplemented with 0.2 % cycloheximide. Dermatophyte test medium (DMT) invariably induced a color change from yellow to red in all tested strains (57). Urease test has been used to distinguish e.g. between *Trichophyton mentagrophytes* and *T. rubrum* (20). Eight *Nannizzia* species tested by Hubka et al. (12) were urease positive. This corresponds partly to our results; negative or weak reactions at one of both time points were recorded in some species. Milk hydrolysis on BCP-MS-G has occasionally been used in dermatophyte diagnostics (22, 23) including *Nannizzia* species. Our results yielded variable reactions for all tested species, from negative to weak and positive. Some strains had a slow response, being recorded as negative on day 7 and positive on day 14 day. In addition of being strain-dependent, the color change was often difficult to evaluate, being concealed by colony growth and pigmentation. Lipase activity on TOTM was reproducible within strains and we found some unambiguous correlation with the previously published results of Elavarashi et al. (1). The latter authors also tested hemolytic activity which was negative in *N. gypsea.* In our study the hemolytic activity at day 7 was negative for all tested species expect *N. aenigmatica*, *N. duboisii* and the majority of *N. nana* strains were positive after 14 days. Hemolytic activity in dermatophytes has been regarded as strain-dependent (58), but in our study all species showed uniform results with this feature, except for a single deviating strain in *N. nana* and *N. duboisii* (Table 3). In general, the diagnostic value of these four tests is limited due to intra-specific variation and reproducibility. Growth at 37 °C is variable within some species as well, diminishing its discriminative power. Previously published results (12) discriminated *N. aenigmatica* and *N. praecox* from remaining species as being unable to grow at this temperature. In our tests, six out of ten strains of *N. fulva* were also negative while the other four strains had poor growth. Poor growth was recorded for two in *N. gypsea*, one in *N. nana* and in *N. duboisii*.

In conclusion, this study on genus *Nannizzia* using phenotypic and molecular identifications has demonstrated that molecular methods are superior and the most reliable. All loci except LSU can be used for identification purposes. Macro and microscopic features and physiology are not very informative; they are still useful for clinics with no molecular facilities. Our results showed that the physiological test are even less informative than anticipated, due to the high intraspecies variation. The features of *Nannizzia* species described in this paper may reveal a higher prevalence of this species in the clinical cases which would be omitted or misidentified, thus helping in establishment of better epidemiological follow-up of these fungi.

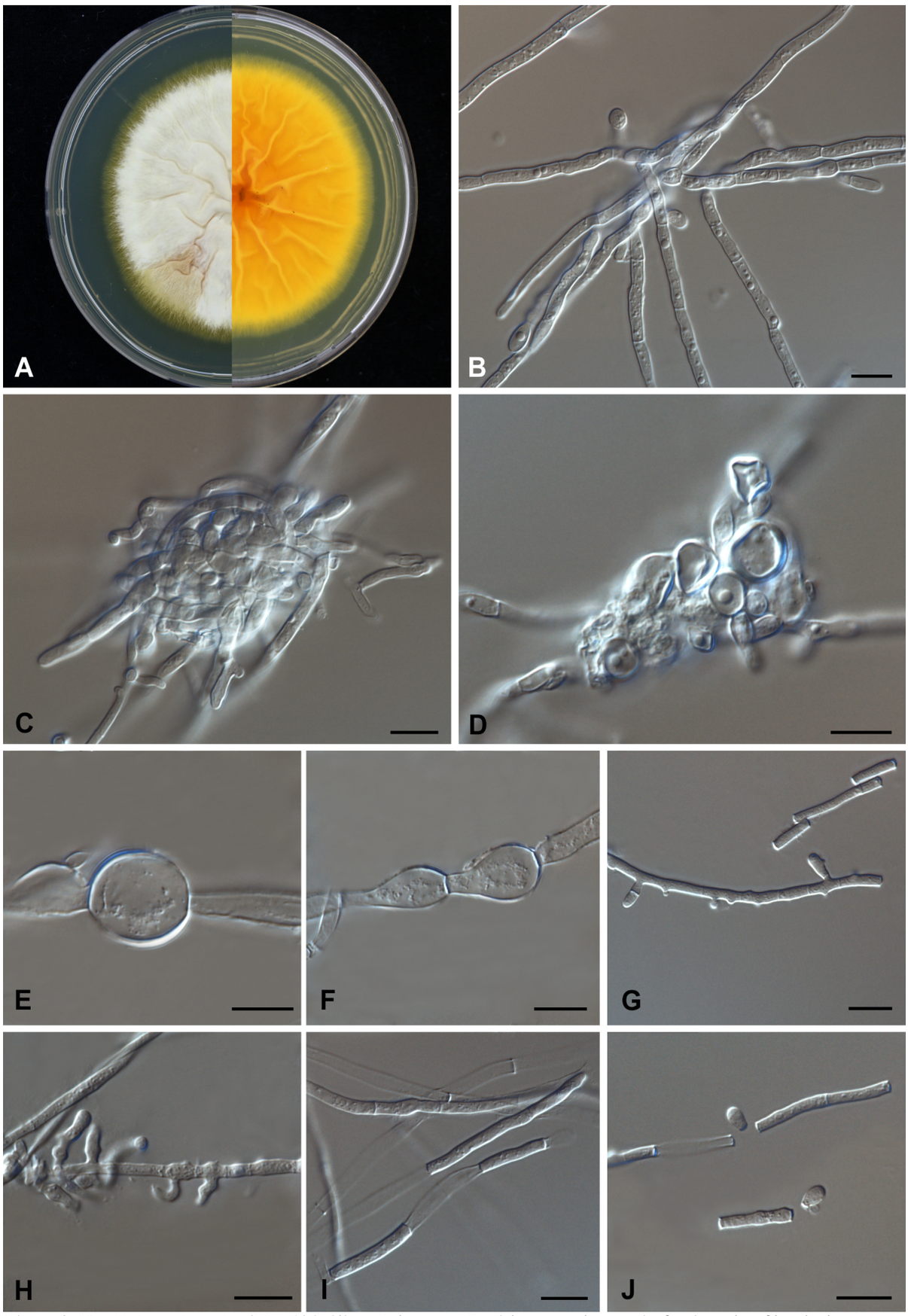

Figure 3. *Nannizzia aenigmatica* (CBS 134549). a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27°C; b–d. Hyphae in clumps; e. Chlamydospore; f. Racquet hyphae; g. Hyphae and arthroconidia; h. Hook-shaped short hyphae. i. Arthroconidia. j. Micro- and arthro- conidia . — Scale bars = 10 µm

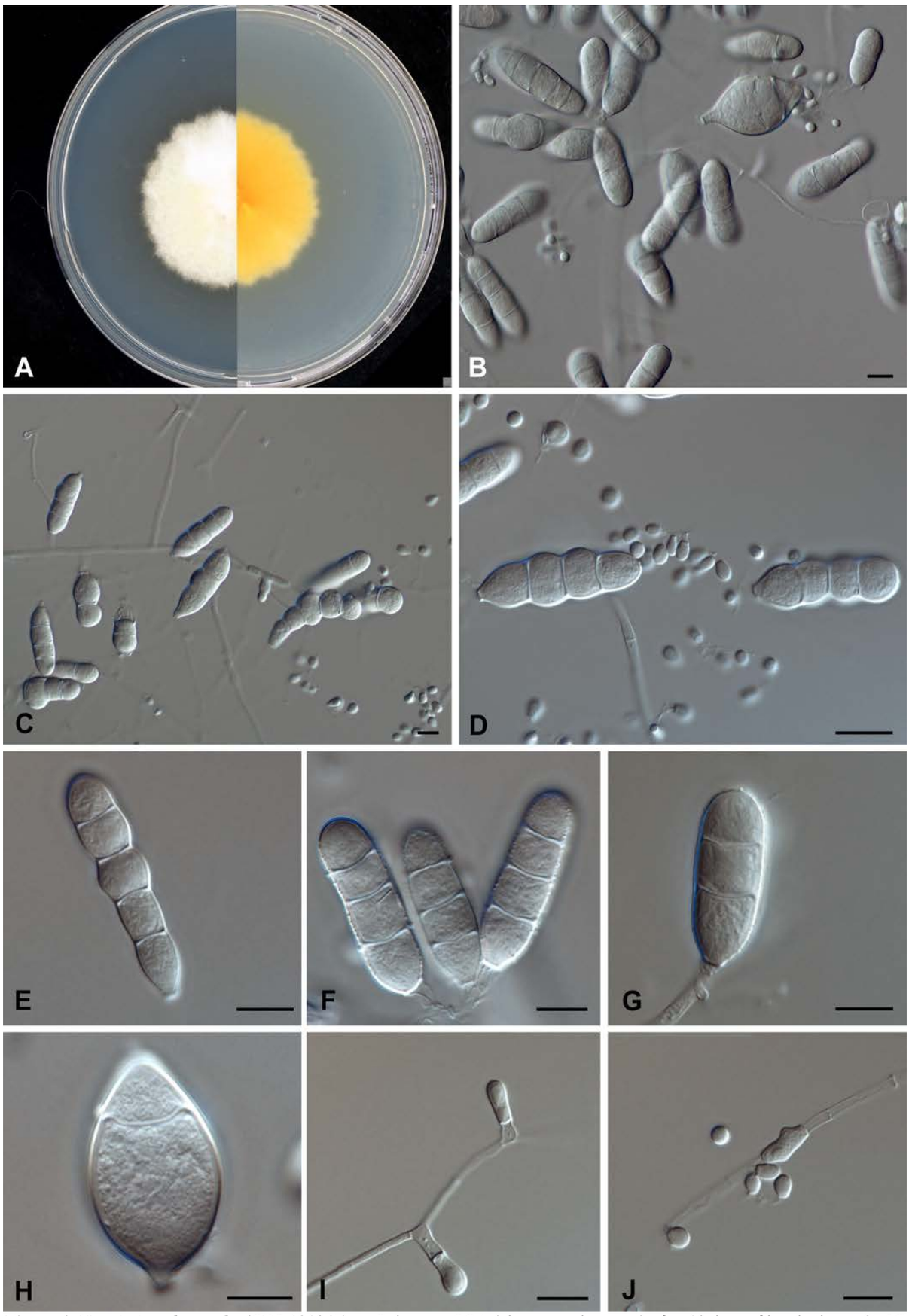

Figure 4. *Nannizzia polymorpha* (CBS 121947). a. Colony on SGA (obverse and reverse) after 10 days of incubation at 27 °C; b–d. Macro- and micro- conidia; e–h. Macroconidia; i–j. Microconidia. — Scale bars = 10 µm

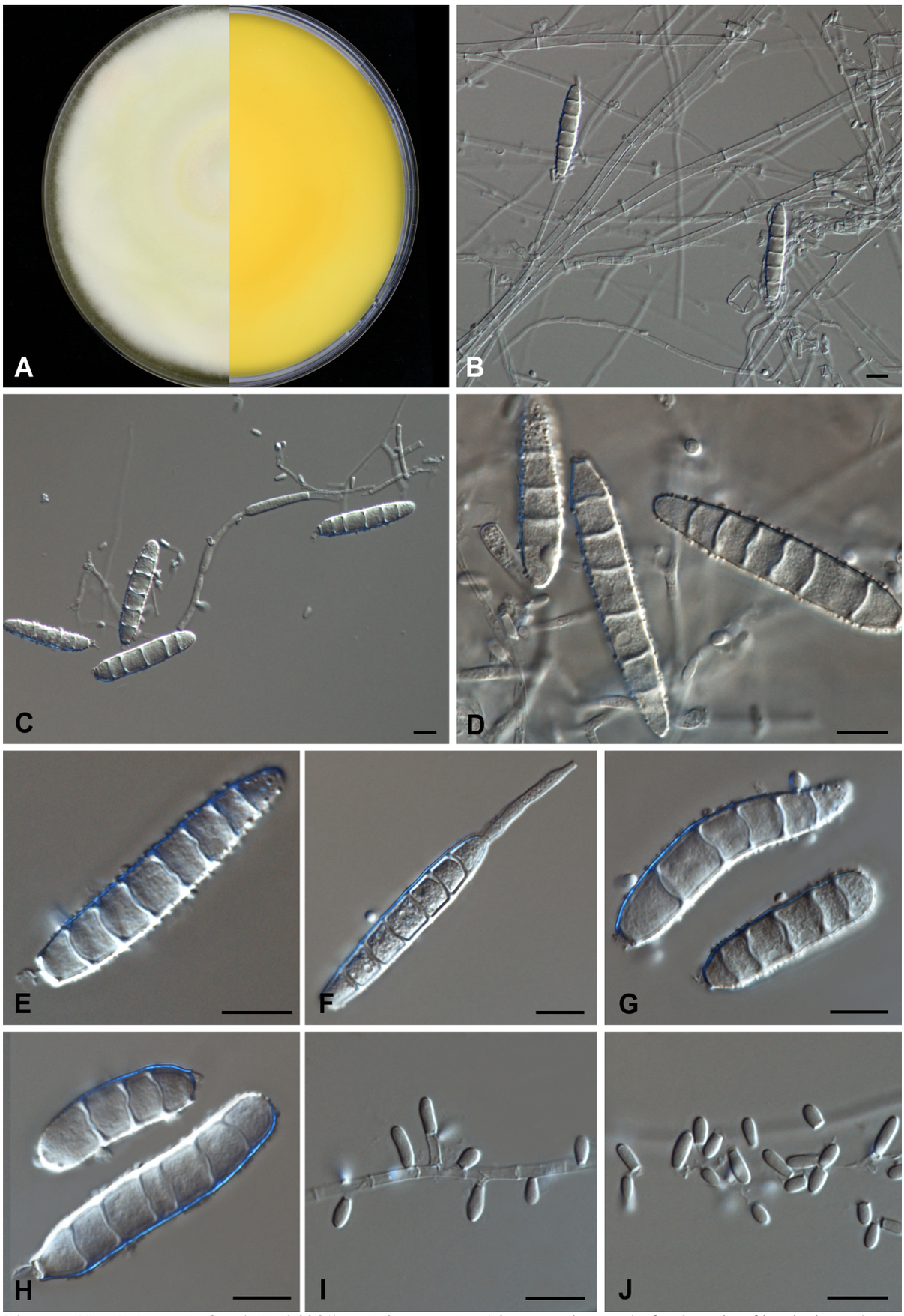

Figure 5. *Nannizzia corniculata* (CBS 366.81). a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27 °C; b–h. Macroconidia; i, j. Microconidia. — Scale bars = 10 µm.

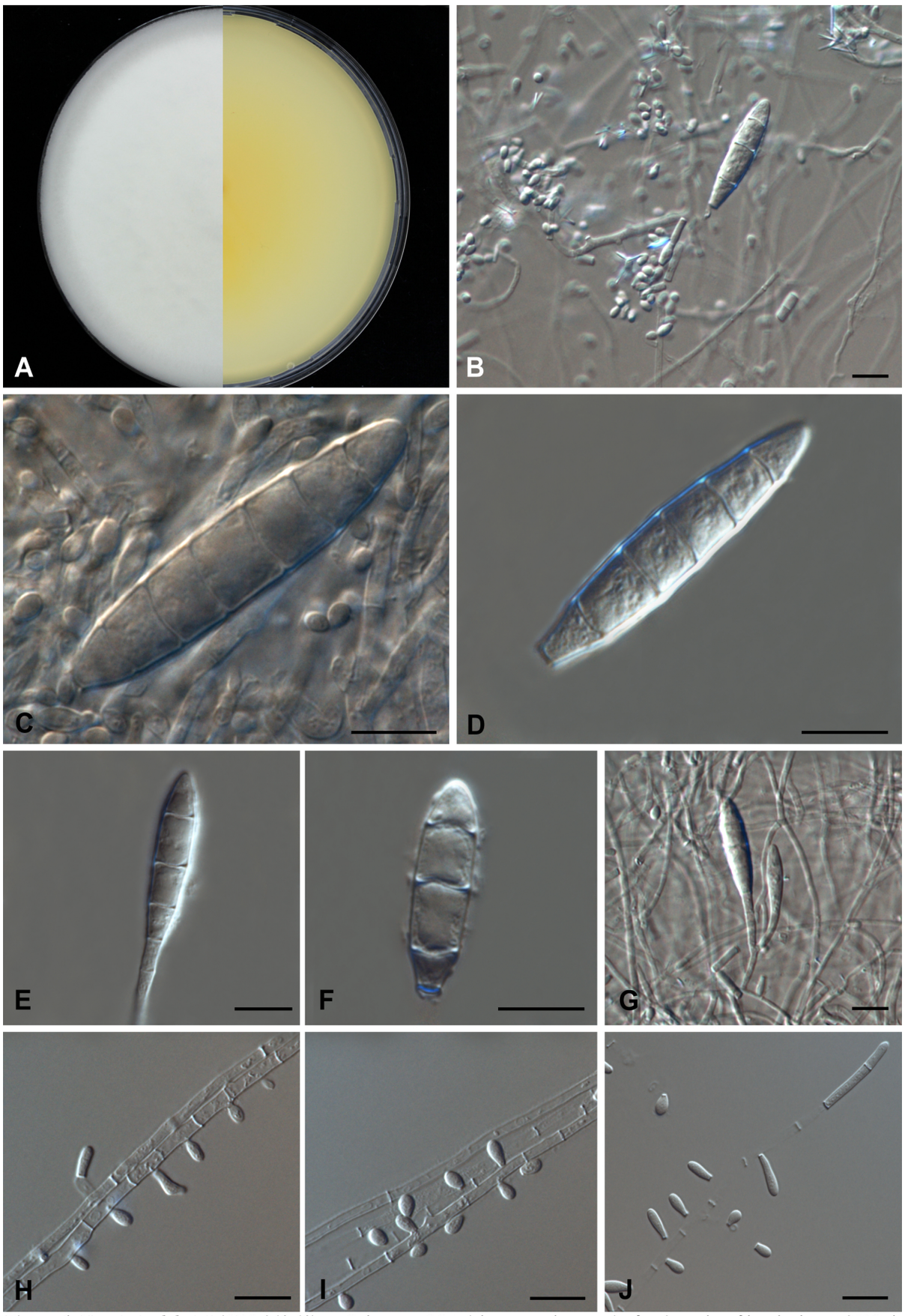

Figure 6. *Nannizzia duboisii* (CBS 349.49). a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27°C; b. Macro- and micro- conidia; c–g. Macroconidia; h–j. Macroconidia. — Scale bars = 10 µm.

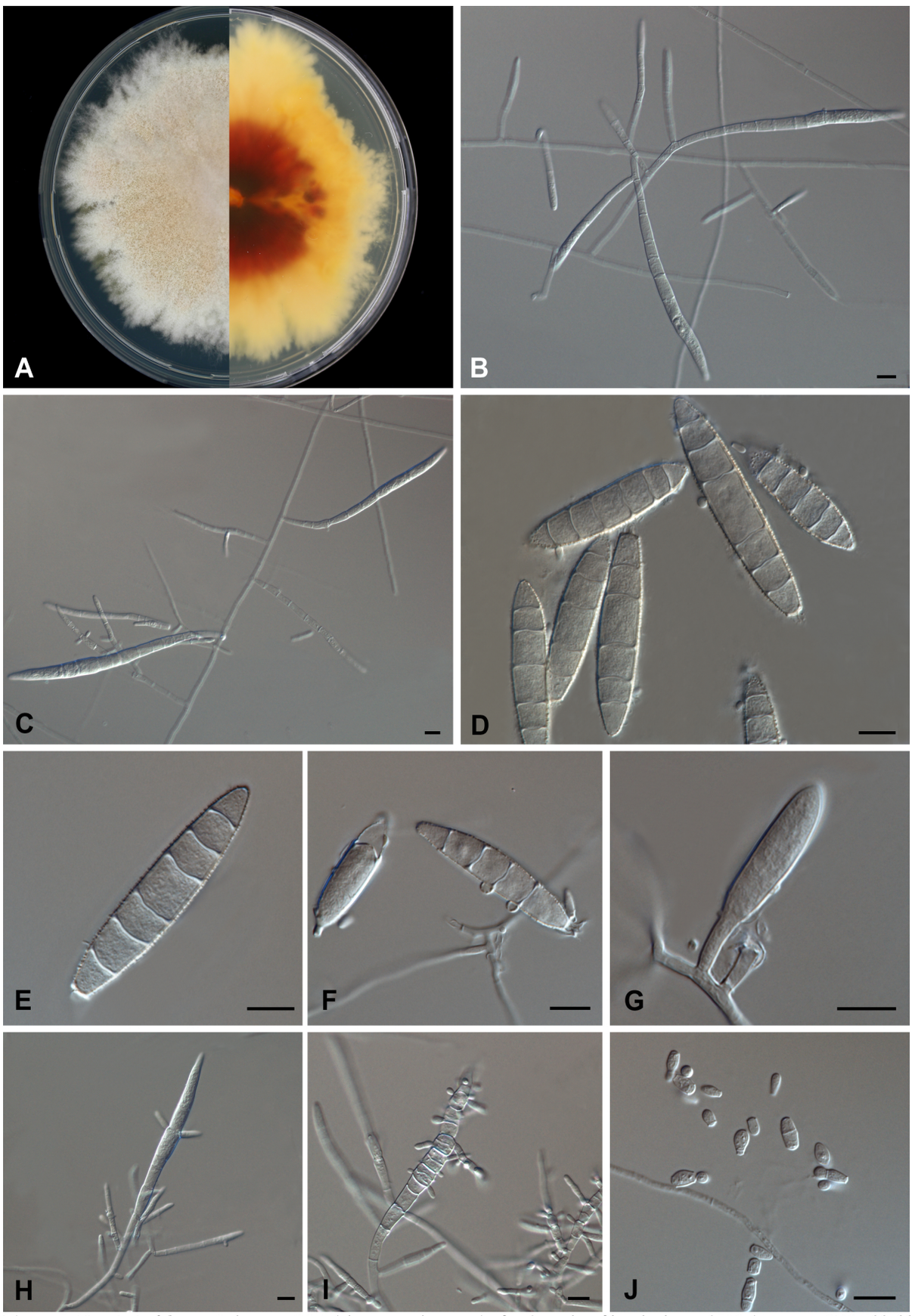

Figure 7. *Nannizzia fulva*. a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27°C (Fac. syn. CBS 599.66 M. boullardii); b, c, h, i. Coherent non-maturating elongated conidia (CBS 599.66); d–g. Mature macroconidia (fac. syn. *Keratinomyces longifusus* CBS 243.64); j. Microconidia (CBS 243.64). — Scale bars = 10 µm.

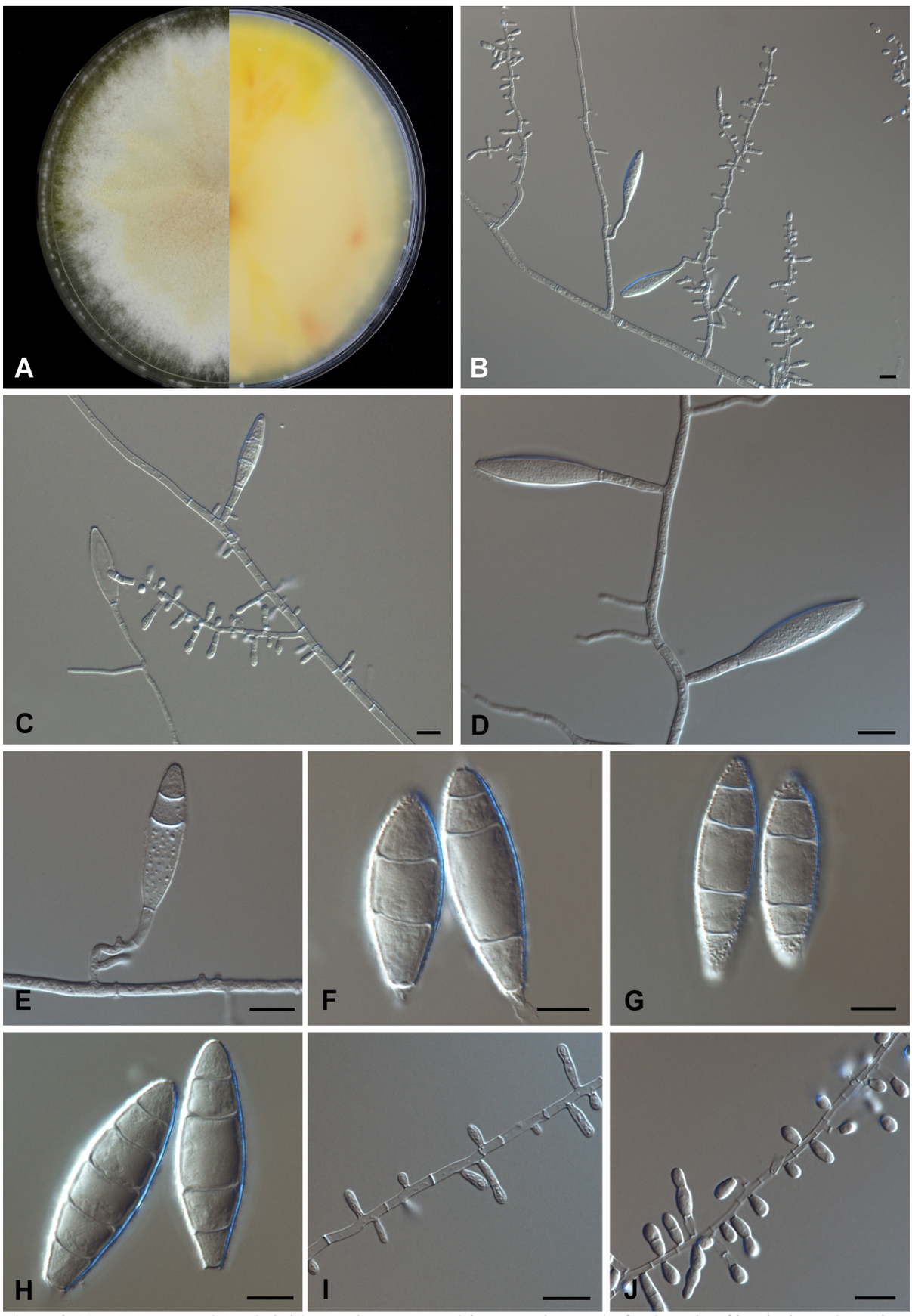

Figure 8. *Nannizzia gypsea* (CBS 258.61). a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27°C; b–c. Macro- and micro- conidia; d–h. Macroconidia; i, j. Microconidia. — Scale bars = 10 µm

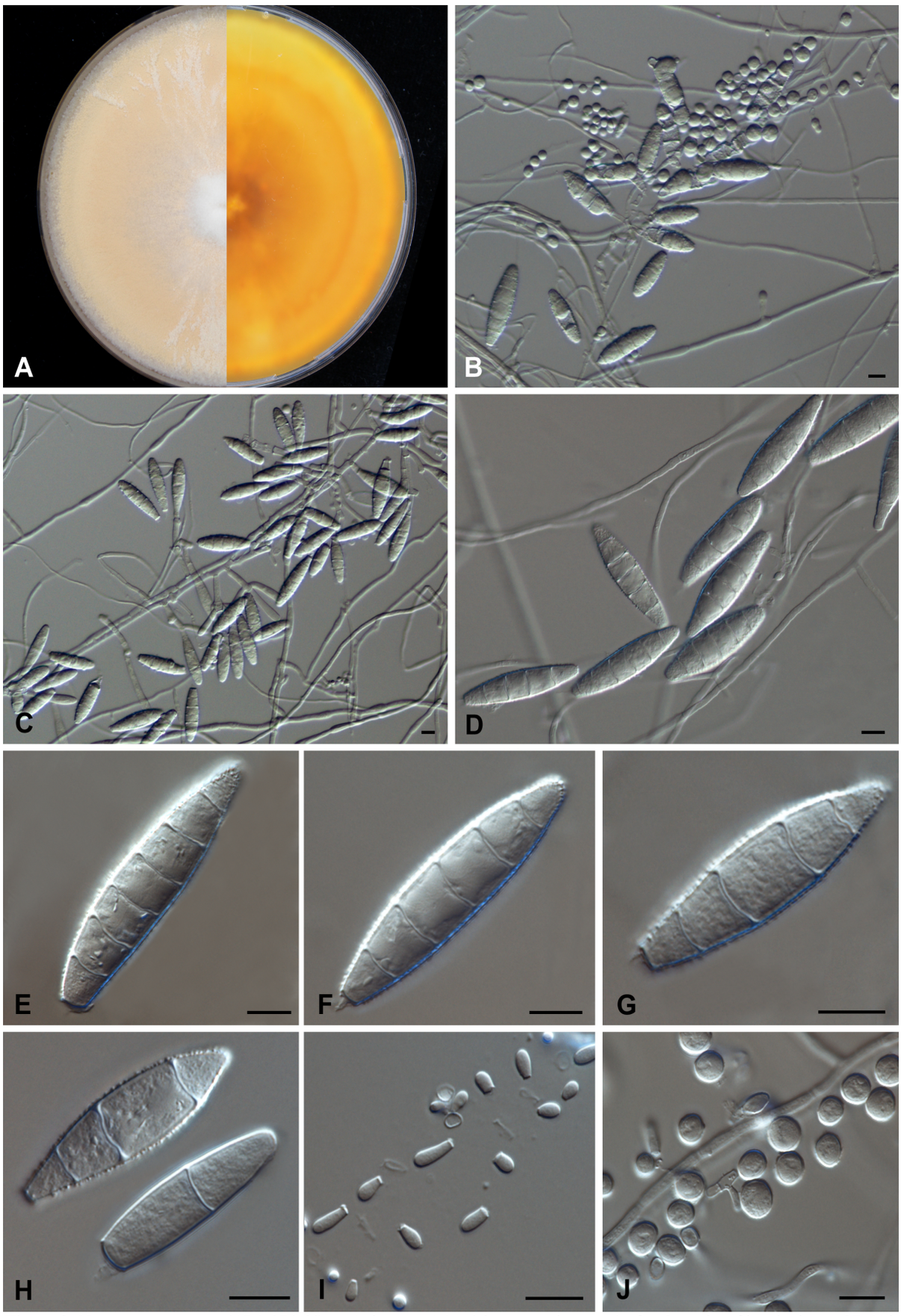

Figure 9. *Nannizzia incurvata* (CBS 174.64). a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27°C; b. Macro- and micro- conidia; c–h. Macroconidia; i, j. Microconidia. — Scale bars = 10 µm

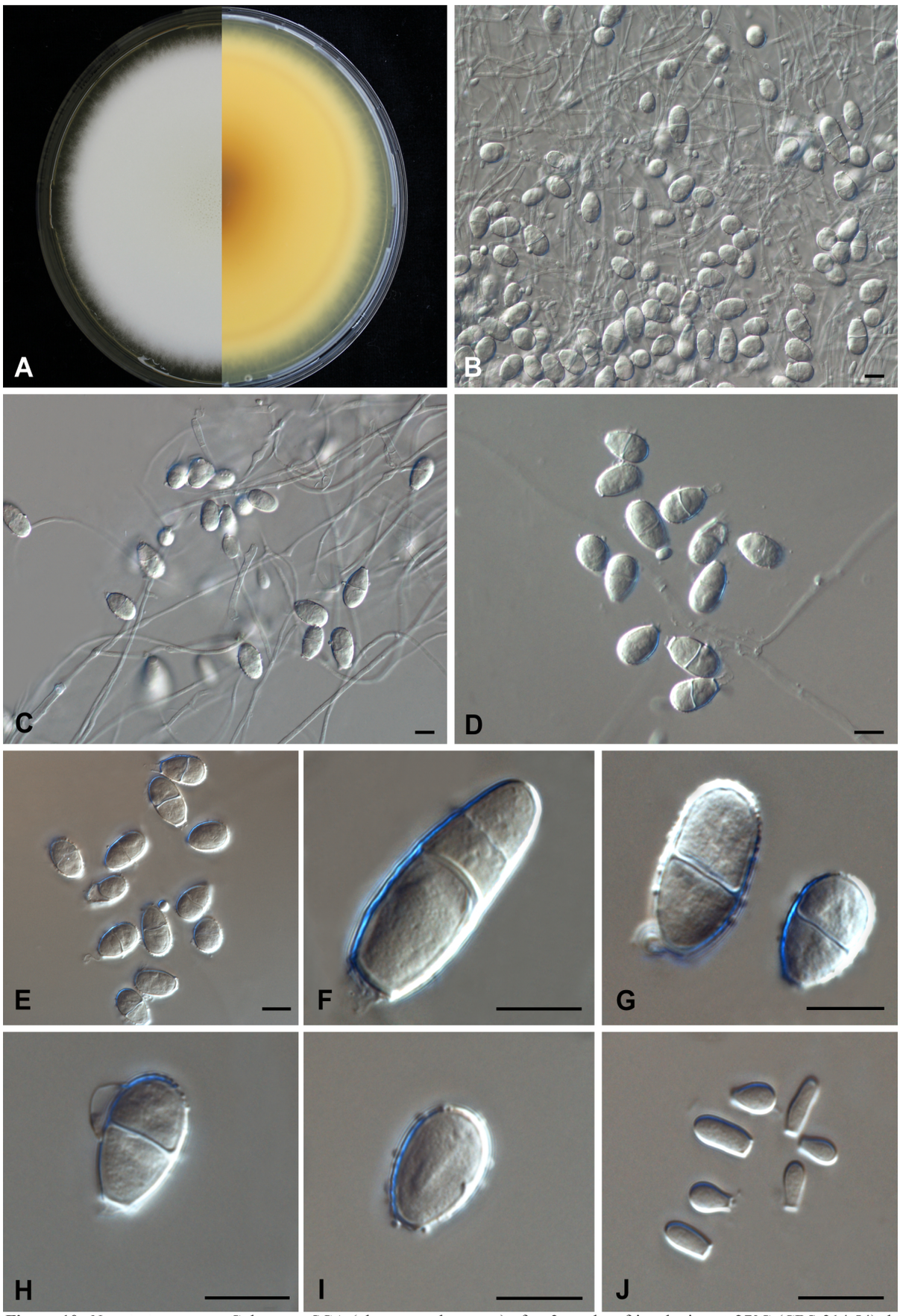

Figure 10. *Nannizzia nana*. a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27°C (CBS 314.54); b. Macro- and micro- conidia; c–i. Macroconidia; j. Microconidia. c–j. (CBS 727.88). — Scale bars = 10 µm

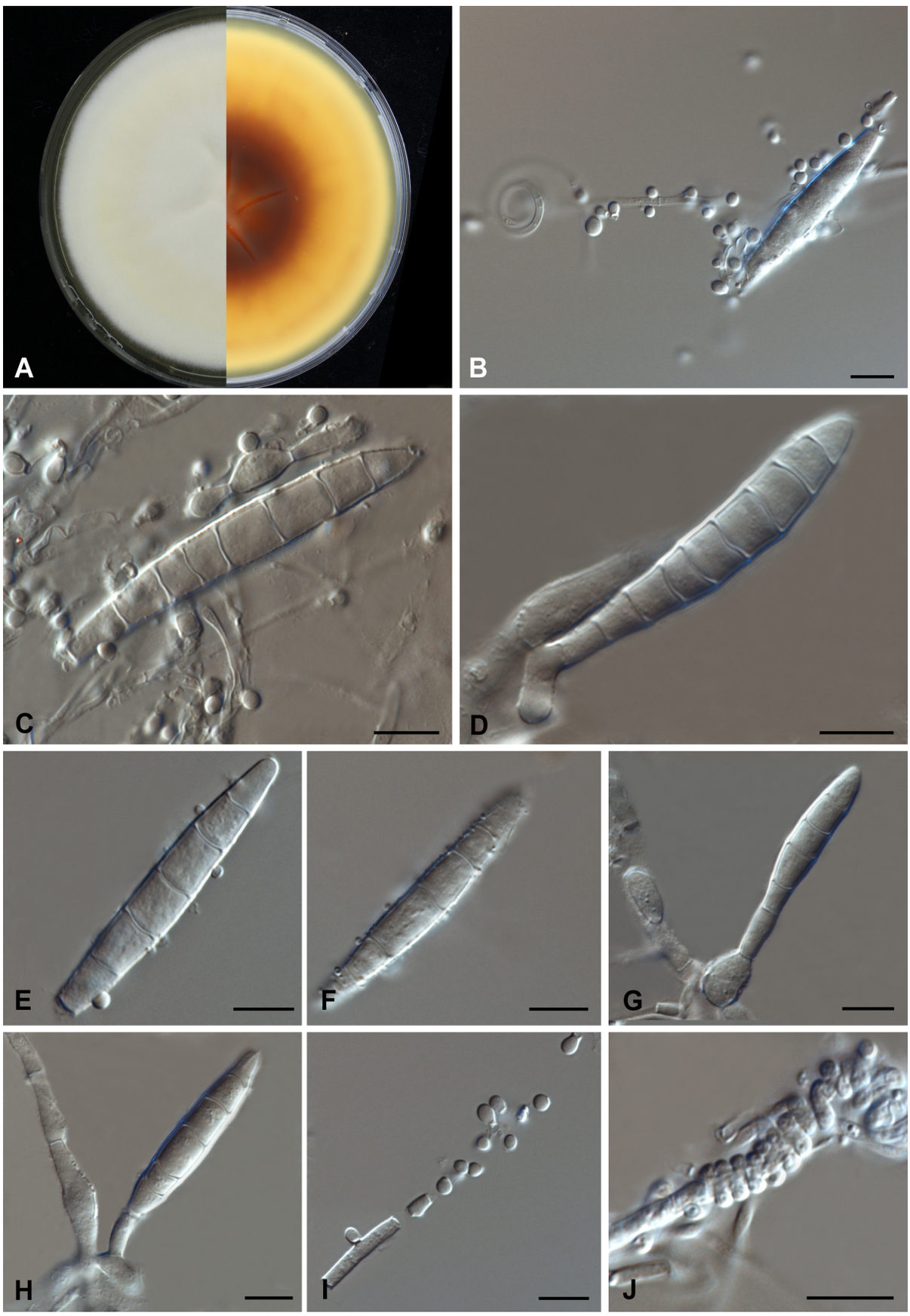

Figure 11. *Nannizzia persicolor*. a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27°C (CBS 871.70); b–c. Macro- and micro- conidia; d–h. Macroconidia; i. Microconidia; j. Spiral hyphae. c–j. (CBS 139323). — Scale bars = 10 µm

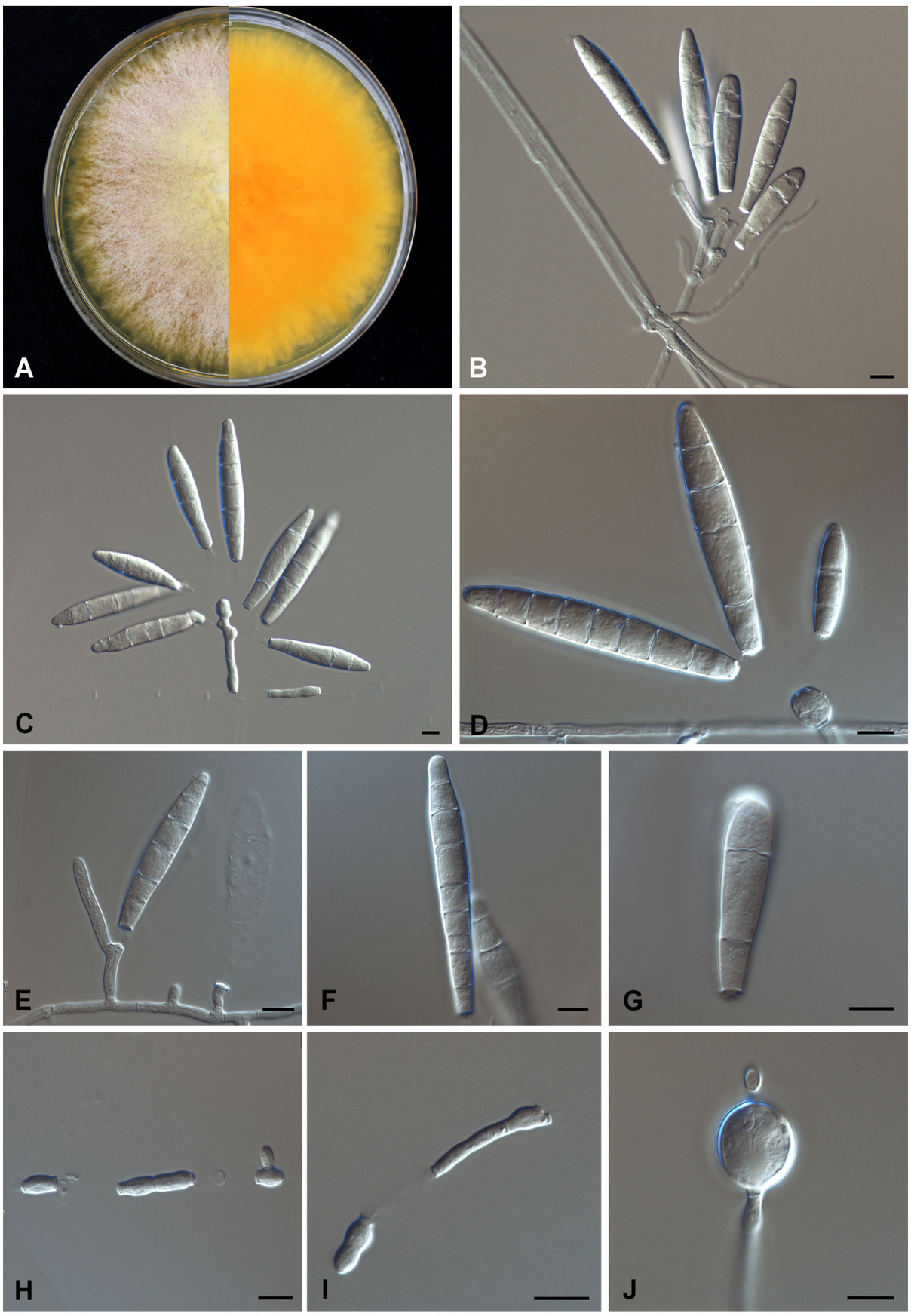

Figure 12. *Nannizzia praecox* (CBS 671.89). a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27°C; b–g. Macroconidia; h. Macro- and arthro- conidia; i. arthroconidia; j. Chlamydospore. — Scale bars = 10 µm.

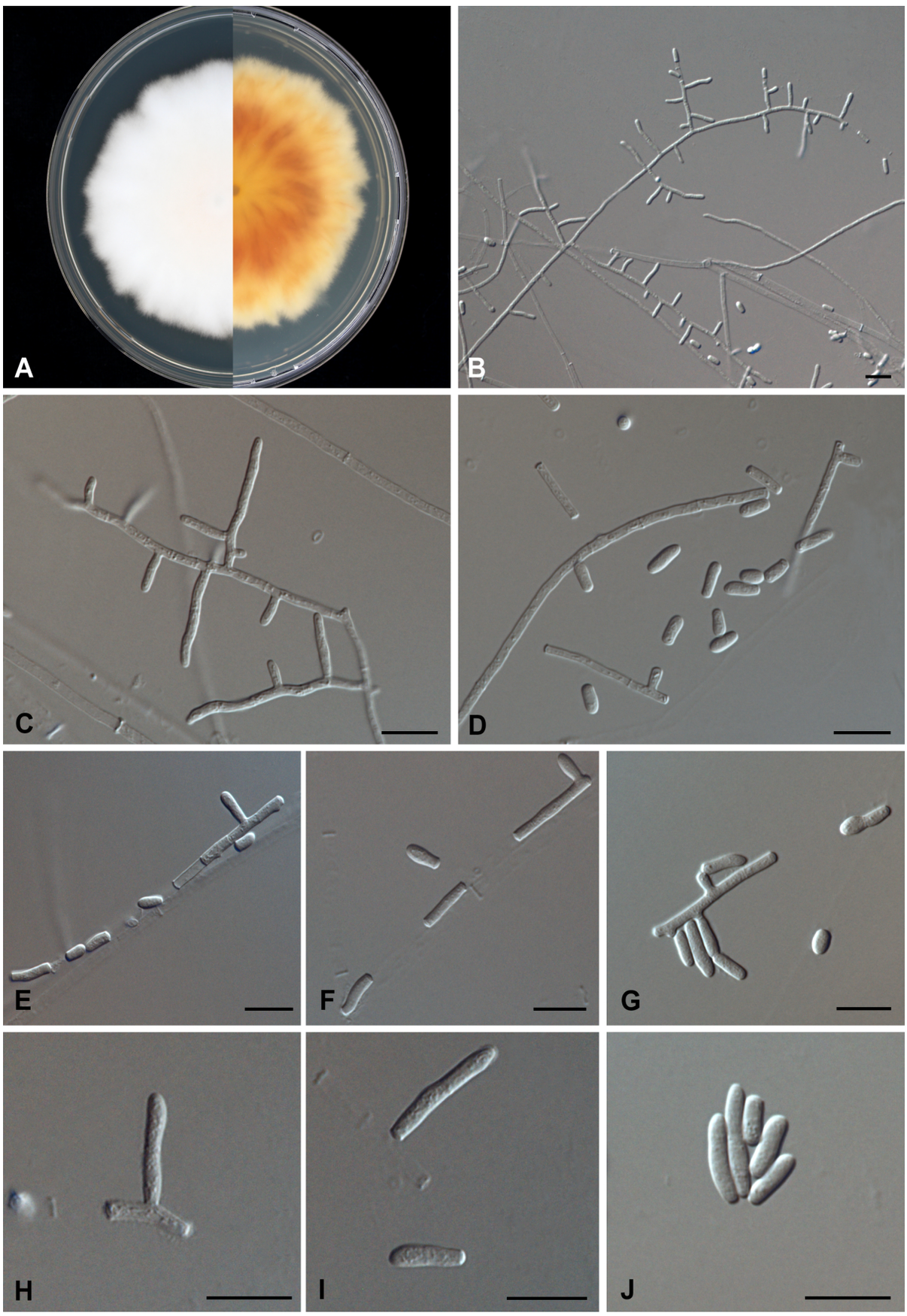

Figure 13. *Nannizzia lorica* (CBS 450.65). a. Colony on SGA (obverse and reverse) after 3 weeks of incubation at 27°C; b–j. Micro- and arthro- conidia. — Scale bars = 10 µm.

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ST1. Sequence similarity for ITS, LSU, *TUB2*, *RP 60S L1* and *TEF3*

ITS	N.ae	N.po	N.co	N.du	N.fu	N.gy	N.in	N.na	N.pe	N.pr
N.po	85%									
N.co	91%	**87%**								
N.du	91%	**87%**	97%							
N.fu	91%	**85%**	95%	93%						
N.gy	92%	**86%**	95%	95%	93%					
N.in	**84%**	**76%**	**83%**	**83%**	**85%**	**83%**				
N.na	**83%**	**78%**	**81%**	**82%**	**84%**	**81%**	**85%**			
N.pe	**89%**	**85%**	93%	93%	**90%**	92%	**81%**	**81%**		
N.pr	92%	**86%**	91%	91%	91%	**90%**	**85%**	**85%**	92%	
N.lo	95%	**84%**	91%	91%	92%	92%	**85%**	**82%**	**89%**	91%

LSU	N.ae	N.po	N.co	N.du	N.fu	N.gy	N.in	N.na	N.pe	N.pr
N.po	98%									
N.co	99%	99%								
N.du	99%	99%	100%							
N.fu	99%	99%	100%	100%						
N.gy	99%	99%	99%	100%	100%					
N.in	99%	99%	99%	99%	99%	99%				
N.na	98%	99%	99%	99%	99%	99%	99%			
N.pe	99%	99%	99%	100%	99%	99%	100%	99%		
N.pr	99%	99%	99%	100%	99%	99%	99%	99%	100%	
N.lo	99%	98%	100%	100%	100%	100%	99%	99%	99%	99%

TUB2	N.ae	N.po	N.co	N.du	N.fu	N.gy	N.in	N.na	N.pe	N.pr
N.po	88%									
N.co	**90%**	**89%**								
N.du	91%	**88%**	**90%**							
N.fu	91%	**88%**	94%	**90%**						
N.gy	95%	**89%**	**90%**	91%	92%					
N.in	91%	**90%**	92%	91%	92%	91%				
N.na	**88%**	91%	**88%**	**88%**	**88%**	**88%**	**89%**			
N.pe	**86%**	**89%**	**89%**	**87%**	**89%**	**86%**	**88%**	**88%**		
N.pr	87%	**89%**	**89%**	**87%**	**90%**	**88%**	**89%**	**88%**	91%	
N.lo	93%	**89%**	**90%**	91%	91%	94%	91%	**88%**	**86%**	**87%**

RB 60S L1	N.ae	N.po	N.co	N.du	N.fu	N.gy	N.in	N.na	N.pe	N.pr
N.po	90%									
N.co	94%	**90%**								
N.du	92%	**89%**	94%							
N.fu	94%	91%	95%	94%						
N.gy	95%	**90%**	94%	92%	93%					
N.in	**90%**	**87%**	**90%**	**87%**	**90%**	**89%**				
N.na	**89%**	**90%**	**89%**	**88%**	**90%**	**88%**	**87%**			
N.pe	**90%**	**88%**	**89%**	**89%**	**89%**	**88%**	**87%**	**90%**		
N.pr	**89%**	**90%**	**89%**	**89%**	**90%**	**88%**	**86%**	**89%**	91%	
N.lo	93%	**88%**	93%	92%	92%	94%	**87%**	**87%**	**87%**	**87%**

TEF3	N.ae	N.po	N.co	N.du	N.fu	N.gy	N.in	N.na	N.pe	N.pr
N.po	93%									
N.co	94%	95%								
N.du	96%	94%	96%							
N.fu	95%	94%	97%	97%						
N.gy	97%	95%	97%	97%	96%					
N.in	96%	93%	94%	95%	94%	95%				
N.na	91%	94%	93%	93%	93%	92%	93%			
N.pe	94%	97%	96%	96%	95%	96%	93%	94%		
N.pr	95%	94%	96%	97%	95%	97%	96%	94%	95%	
N.lo	96%	94%	96%	97%	96%	97%	97%	93%	95%	98%

Legend: **bold ≤ 90 %,** underlined, 91–95 %, N.ae = *N. aenigmatica*, N.po = *N. polymorpha*, N.co = *N. corniculata*, N. du = *N. duboisii*, N.fu = *N. fulva*, N. gy = *N. gypsea*, N.in = *N. incurvata*, N.na = *N. nana*, N.pe = *N. persicolor*, N.pr = *N. praecox*, N.lo = *N. lorica.*

CHAPTER 5

Novel taxa of thermally dimorphic systemic pathogens in the *Ajellomycetaceae* (*Onygenales*)

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Abstract

Recent discoveries of novel systemic fungal pathogens with thermally dimorphic yeast-like phases have challenged the current taxonomy of the *Ajellomycetaceae*, a family currently comprising the genera *Blastomyces*, *Emmonsia*, *Emmonsiellopsis, Helicocarpus, Histoplasma, Lacazia* and *Paracoccidioides*. Our morphological, phylogenetic and phylogenomic analyses demonstrated species relationships and their specific phenotypes, clarified generic boundaries and provided the first annotated genome assemblies to support the description of two new species. A new genus, *Emergomyces,* accommodates *Emmonsia pasteuriana* as type species, and the new species *Emergomyces africanus,* the aetiological agent of case series of disseminated infections in South Africa. Both species produce small yeast cells that bud at a narrow base at 37 °C and lack adiaspores, classically associated with the genus *Emmonsia*. Another novel dimorphic pathogen, producing broad-based budding cells at 37 °C and occurring outside North America, proved to belong to the genus *Blastomyces*, and is described as *Blastomyces percursus*

Keywords

Ajellomycetaceae, *Blastomyces*, *Emergomyces*, *Emmonsia*, genomics, phylogeny

Introduction

Recent discoveries of novel systemic human pathogens with a thermally dimorphic pathogenic phase that consist of budding yeast cells have challenged the current taxonomy of the family *Ajellomycetaceae,* order *Onygenales* (1). For nearly 100 years, only four genera of classical systemic pathogens, each containing just one or two species, were recognised in the order *Onygenales*, ie *Coccidioides*, *Blastomyces*, *Histoplasma* and *Paracoccidioides*. All these fungi reside in their filamentous forms in soil or guano, and upon inhalation by the host, they morphologically shift to an invasive spherule (*Coccidioides*) or a yeast-like form in the host's pulmonary system. While phylogenetic analyses have placed the genus *Coccidioides* in the family *Onygenaceae*, *Blastomyces, Histoplasma* and *Paracoccidioides* proved to be members of the family *Ajellomycetaceae* (2).

Another documented genus within the *Ajellomycetaceae* is *Emmonsia*, until recently known mainly for species that cause pulmonary infections in small mammals. Until the description of *Emmonsia pasteuriana* in 1998, the genus *Emmonsia* contained two species: the genetically homogeneous *Emmonsia crescens* and a more diverse species, *Emmonsia parva* (3). These are the aetiological agents of adiaspiromycosis, a pulmonary disease of terrestrial mammals and occasionally of humans (4, 5). They differ from classical dimorphic pathogenic fungi by their pathogenic phase consisting of large, thick-walled adiaspores instead of budding yeast cells. *Emmonsia crescens* has adiaspores often over 100 µm in diameter and a maximum growth temperature of 37 °C, while the adiaspores of *Emmonsia parva* are mostly 15-25 µm in diameter and the fungus grows up to 40 °C. Multilocus phylogenetic analysis suggested these species to be less closely related than anticipated. *Emmonsia parva* clustered with *Blastomyces dermatitidis/B. gilchristii*, while *Emmonsia crescens* took a rather isolated position (1). Recent phylogenomic analysis supported *Emmonsia crescens* as a sister group to the clade including *Histoplasma*, *Emmonsia parva* and *B. dermatitidis/B. gilchristii* (6). Taken together, the genetic evidence suggests that, in spite of striking morphological, ecological and pathophysiological similarities, aetiological agents of adiaspiromycosis are polyphyletic.

Since the 1970s, novel pathogens have emerged with phylogenetic, morphological and clinical similarities to known members of the *Ajellomycetaceae.* Schwartz et al. (1) summarised reports of numerous additional human cases due to novel species in the family *Ajellomycetaceae*, most of which remained undescribed. Recently, Wang et al. (7) reported on another novel species from China. Several of these novel taxa are opportunistic pathogens of immunocompromised hosts, primarily persons infected with HIV (1). An important emerging species associated with disease in advanced HIV infection was found in South Africa with at least 56 cases reported since being correctly identified in 2008 (8, 9). The agent causing this mycosis was closely related to *Emmonsia pasteuriana*, which is also known to infect patients with AIDS and patients with other immune disorders (10-12).

The present work studies the relationships of unclassified isolates from clinical sources with existing *Emmonsia* and *Blastomyces* species and other members of the *Ajellomycetaceae* by means of comprehensive morphological and phylogenetic analyses involving both multilocus and whole genome sequencing. We describe the new genus *Emergomyces* to include *Emmonsia pasteuriana* as type species (*Emergomyces pasteurianus* comb. nov.) and *Emergomyces africanus* sp. nov. for a fungus formerly mentioned by Schwartz et al. as *Emmonsia* sp. 5 (1). Another dimorphic human pathogen is described as *Blastomyces percursus* sp. nov. (formerly named *Emmonsia* sp. 3 (1). We completed the first annotated genome assemblies for these novel species, which may guide the development of new diagnostics.

Table 1. Strains used in this analysis and shown in phylogenetic trees. Only the sequences from the taxa described in this paper were submitted to GenBank.

Taxon name	CBS accession	Other collections	Source	Country	References	Genbank Nr.(ITS, LSU, TUB2, TEF3, RP60SL1)	GenBank genome Nr.
B. dermatitidis	CBS 674.68 (T)	ATCC 18188; B788(a); IHEM 3783; UAMH 3539	human, blastomycosis	USA	(6)		ADMK00000000
B. dermatitidis		ER-3 (ATCC MYA 2586)	soil under woodpile	USA	(6]		ACBT00000000
B. gilchristii	CBS 134223 (T)	SLH14081	human, blastomycosis	Canada	(6)		ACBU00000000
B. percursus	CBS 139878 (T)	UAMH 7425	human, immunocompetent, mucocutaneous	Israel	(3,47)	KY195964, KY195971, KY195936, KY195942, KY195949	
B. percursus		BP222	human, immunocompetent, brain abscess / pneumonia	South Africa	(24)		LGTZ00000000
B. percursus		NCPF 4091	human, disseminated / cutaneous	South Africa	(9)	KY195963, KY195972, KY195940, KY195943, X	
Blastomyces sp. 1	CBS 139874	UAMH 3398	human, alcoholic / diabetes, meningo-encephalitis	Canada	(1,36)		
Blastomyces sp. 2*	CBS 139879	UAMH 139	weasel	USA	(3,6)		LDEV00000000
Ea. parva	CBS 139881	UAMH 130	rodent lung	USA	(3]		
Es. africanus	CBS 136260 (T)		human, HIV, disseminated	South Africa	(9)		LGUA00000000
Es. africanus	CBS 136730		human, HIV, disseminated	South Africa	(9)	KY195959, KT155137, KT155489, KT156143, X	
Es. africanus	CBS 140362		human, HIV, disseminated	South Africa	(9)	KY195961, KY195968, KY195939, X, KY195952	

Es. africanus	CBS 140363		human, HIV, disseminated	South Africa	(9)	KY195958, KY195965, X, X, KY195954	
Es. africanus	NCPF 4164		human, HIV, disseminated	South Africa	(9)	KY195960, X, KY195941, KY195945, KY195948	
Es africanus	CBS 140360		human, HIV, disseminated	South Africa	(9)	KY195957, KY195967, KY195937, X, KY195955	
Es africanus	CBS 139543		human, HIV, disseminated	South Africa	(9)	KY195956, KY195966, KY195935, KY195944, KY195953	
Ea. crescens	CBS 177.60 (T)	ATCC 13704; UAMH 3008	rodent, lung	Norway	(3,42,43)		LCZI00000000
Es. pasteurianus	CBS 101426 (T)	IP 2310.95; UAMH 9510;NCPF 4236	human, HIV, disseminated	Italy	(10,25)		LGRN00000000
Es. pasteurianus	CBS 140361		human	South Africa	This study	KY195962, KY195969, KY195938, KY195946, KY195950	
Es. pasteurianus	CBS 139522	F003	human, renal transplant, disseminated	China	(45)	KT155632, KT155632, KY195934, KY195947, KY195951	
Es. orientalis	CBS 124587		human, diabetes, disseminated	China	(7)		
Emergomyces sp. 2	CBS 139872	UAMH 7172	human, HIV, disseminated	Canada	(3]		
Emergomyces sp. 2	CBS 139873	UAMH 10370	human, transplant, disseminated	Canada	(40)		
Emergomyces sp. 6	CBS 102456	UAMH 10427	human, rheumatic / corticosteroids, pulmonary	Germany	(38)		

* Formerly *Emmonsia parva*. Abbreviations used: B = *Blastomyces*, Ea = *Emmonsia*, Es = *Emergomyces*, T = type strain, CBS = CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; NCPF = National Collection of Pathogenic Fungi, Bristol, England; UAMH = Centre For Global Microfungal Biodiversity

Materials and methods

Strains and phenotypes

Reference strains were taken from the collection of the Centraalbureau voor Schimmelcultures (CBS) of CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, the University of Alberta Microfungus Collection and Herbarium (UAMH), Devonian Botanic Garden, Edmonton, Canada (now UAMH Centre for Global Microfungal Biodiversity, Toronto, Canada) and the National Collection of Pathogenic Fungi (NCPF), Mycology Reference Laboratory, Bristol, U.K., supplemented by kind donations of individual researchers. Twenty-four strains were selected for detailed morphological and molecular study (Table 1). These were part of a larger dataset comprising 109 strains, including outgroup (data not shown) used in multilocus analyses. Reference strains belonging to *Coccidioides, Paracoccidioides*, *Blastomyces* and *Histoplasma* as well as the novel taxa described here were handled in biosafety level 3 (BSL-3) laboratories; *Emmonsia crescens* and *Emmonsia parva* were handled at BSL-2. Strains were cultured on 2% Malt Extract Agar (MEA, Oxoid) plates using inoculum from lyophilised, cryo-preserved or fresh mycelium. Cultures were incubated for 28 days at a temperature of 24 °C.

Microscopic observations were done with slide cultures using MEA, as an optimal medium for conidium formation. Agar blocks of ~0.5 cm^2 were placed on agar plates and inoculated at the four sides. The block was subsequently covered with a sterile cover slip (~2 cm^2). Plates were incubated at 24 °C for 7, 14, 21 and 28 days in a closed plastic box with sterile gauze soaked with 5 ml sterile water to avoid drying of the culture. Slides were made by Shear's mounting medium without pigments. Micrographs were taken using a Nikon Eclipse 80i microscope and DS Camera Head DS-Fi1/DS-5m/DS-2Mv/DS-2MBW using NIS-ELEMENT freeware package (Nikon Europe, Badhoevedorp, The Netherlands). Dimensions were determined with the Nikon Eclipse 80i measurement module and the mean and standard deviation were calculated from measurements of 50 conidia.

Cardinal temperatures were determined on MEA at 5, 15, 21, 24, 27, 30, 33, 36, 37, 40 and 42°C. Growth rates were determined in triplicate after 4 weeks incubation. Thermal dimorphism was evaluated by incubation on MEA and Brain Heart Infusion agar plates (BHI, BD Difco) for 1 to 4 weeks, using temperature switch from 24 to 37 °C as sole stimulus for transition (13).

DNA extraction, PCR and sequencing

Fungal material was harvested for DNA extraction using MasterPure™ Yeast DNA Purification Kit from Epicentre (Madison, WI, USA). Five gene regions were amplified. The first two loci were ITS and LSU of the rDNA operon (14). The universal fungal locus ITS1-5.8-ITS2 of the rDNA was amplified with primers ITS5 (15) and ITS4 operated under standard PCR conditions (14). Partial LSU of the rDNA operon was amplified using LR0R and LR5 primer set (16) under the same PCR conditions but with cycle extension of 90 s. Partial β-tubulin (*TUB2*) covering the variable 5'-end containing four small introns, was amplified with TUB2Fd and TUB4Fd primer set (17) partial gene encoding elongation factor 3 (*TEF3*) with Al50+51_EF3_2900_F and Al50+51_EF3_3300_R primer set, and 60S ribosomal protein L10 (*RP 60S L1*) with AlGr52_412-433_F1 and Algr52_1102_1084_R1 primer set (18). Primers and PCR protocols were designed and tested for the development of potential secondary fungal barcodes (18). PCR products were visualised on 1% agarose gels. Positive PCR products were sequenced in cycle-sequencing reaction using ABI big

dye terminator v.3.1 with a modified manufacturer's protocol (18). Following the cycle-sequencing reaction, a capillary electrophoresis system (DNA analyser, Thermo Fisher Scientific, Waltham, USA) was used for performing bidirectional sequencing. Sequences obtained were manually edited and consensus sequences stored in a Biolomics database (19).

Sequence alignment and phylogenetic analysis

Obtained sequences were aligned with MAFFT v. 6.850b with default settings except for the 'genafpair' option (20). Datasets for the five loci were assembled in a single multilocus dataset using SEQUENCE MATRIX software (21). For both the ITS and multilocus datasets, a maximum likelihood phylogeny was inferred using RAxML v.8.0.0 employing GTRCAT model and 1000 bootstrap replicates (22). Bootstrap branch support above 70% was considered as significant. Multiple sequences of species and genera outside the focus of our analysis were collapsed or represented by one or two strains. The multilocus dataset was additionally analysed by Markov chain Monte Carlo (MCMC) algorithm with MRBAYES v. 3.2.6 (23) on the CIPRES portal (http://www.phylo.org) with four simultaneous runs for 10 million generations, with a sampling frequency of 1000 trees. A burn-in tree sample of 25% was discarded. Bayesian posterior probabilities from 50% majority-rule consensus trees with a probability value higher than 0.80 were considered as significant.

Genome sequencing and de novo assembly

Three strains were selected for genome sequencing, including *Ea. pasteuriana* CBS 101426, and BP222 and CBS 136260 initially mentioned as *Emmonsia* sp. 3 (1) and *Emmonsia* sp. 5 (1) respectively (Table 1). Genomic DNA of strain BP222, isolated from a brain abscess in an immunocompetent person in South Africa (24) was extracted and a library with insert sizes ranging from 500 to 1500 bp was sequenced on the Illumina MiSeq platform to obtain paired-end reads of 300 bp. Strain CBS 136260, isolated from a skin biopsy in an HIV-infected patient (9) was sequenced using IonTorrent, generating unpaired reads from 8 to 361 bp. For strain CBS 101426 (=UAMH 9510=NCPF 4236) of *Ea. pasteuriana*, isolated from cutaneous lesions in an Italian woman with advanced HIV infection (10, 25) 100 ng of genomic DNA was sheared to approximately 250 bp for library construction, using a Covaris LE instrument and prepared for sequencing as previously described (26). A library with 180-base inserts was constructed and sequenced on an Illumina HiSeq 2000 platform to generate 101 bp paired-end reads, producing average genome coverage of 191X.

Both Illumina and IonTorrent reads of BP222 and CBS 136260 were assembled using the SPAdes assembler v3.1.1. (27). Next, Pilon v1.16 (28) was used to correct the best assembly from each species, resolving single nucleotide errors (SNPs), artifactual indels and local mis-assemblies, as previously described for *Paracoccidioides* species (29). The 101-bp Illumina reads of *Ea. pasteuriana* were assembled using ALLPATHS-LG (30) with default parameters. All three de novo assemblies were evaluated using gaemr package (http://www.broadinstitute. org/software/gaemr/), which revealed no aberrant regions of coverage, GC content or unexpected sequence similarity suggestive of contamination. Scaffolds representing the mitochondrial genome were separated out from the nuclear assembly.

Gene prediction and annotation

Genes were predicted and annotated by combining calls from multiple methods to obtain the best consensus model for a given locus. These included ab initio predictions (GlimmerHMM, Augustus, Snap, GeneMark-ES), homologous inference (Genewise, TBlastN) and gene model consolidation program EVIDENCEMODELER (31). For protein-coding gene name assignment, we combined HMMER PFAM/TIGRFAM, Swissprot and Kegg products. Kinannote was used to annotate protein kinases (32). To evaluate the completeness of predicted gene sets, the representation of highly conserved genes in a wide range of eukaryotic taxa (core eukaryotic genes; CEGs) were analysed using CEGMA genes (33) with the CoreAlyze tool (http://sourceforge.net/ projects/corealyze/).

Identification of orthologs and phylogenomic analysis

To examine the phylogenetic relationship of novel sequenced species relative to other dimorphic fungi, single-copy orthologs of species from the family *Ajellomycetaceae* were determined and clustered using ORTHOMCL version 1.4 (34) with a Markov inflation index of 1.5 and a maximum e-value of 1e-5. A total of 19 genomes from the *Onygenales* order and three *Aspergillus* genomes were chosen for comparative analyses. These include the following genomes: *Blastomyces gilchristii* SLH 14081 (ACBU00000000), *Blastomyces dermatitidis* ATCC 26199 (AEII00000000), *B. dermatitidis* ATCC 18188 (ADMK00000000), *B. dermatitidis* ER-3 (ACBT00000000), *Emmonsia parva* UAMH 139 (LDEV00000000) and *Ea. crescens* UAMH 3008 (LCZI00000000), *Histoplasma capsulatum* WU24 (AAJI01000000), *H. capsulatum* G186AR (ABBS01000000), *Paracoccidioides lutzii* Pb01 (ABKH02000000), *P. brasiliensis* Pb03 (ABHV02000000), *P. brasiliensis* Pb18 (ABKI02000000), *Coccidioides immitis* RS (AAEC00000000), *C. posadasii* CBS 113859=Silveira (ABAI02000000), *Uncinocarpus reesii* UAMH 1704 (AAIW00000000), *Microsporum gypseum* CBS 118893 (ABQE00000000), *Trichophyton rubrum* CBS 118892 (ACPH01000000), *Aspergillus nidulans* FGSC A4 (AACD00000000), *A. flavus* NRRL3357 (AAIH00000000), *A. fumigatus* Af293 (AAHF01000000). To estimate the species phylogeny, orthologs present in a single copy (1:1) in all of 22 genomes were identified. Multiple protein sequence alignment was performed for each single copy ortholog cluster using MUSCLE to generate sequence alignments of the same length. Then, the cluster multiple alignments were concatenated, and a phylogeny was estimated using RAXML v7.7.8 (22) with model PROTCATWAG with a total of 1000 bootstrap replicates.

Data availability statement

The assemblies and annotations of the described species genomes were deposited at DDBJ/ ENA/GenBank under the following accession numbers: strain BP222 (PRJNA284520), strain CBS 101426 (PRJNA234734) and strain CBS 136260 (PRJNA284519).

Results

Multilocus phylogeny

To examine the phylogenetic relationships of the novel species, we used a panel of 107 strains from the family *Ajellomycetaceae* and multilocus phylogenetic analysis of concatenated sequences of ITS, LSU, *TUB2, TEF3* and *RP 60S L1*. Using *Coccidioides* species (fam.

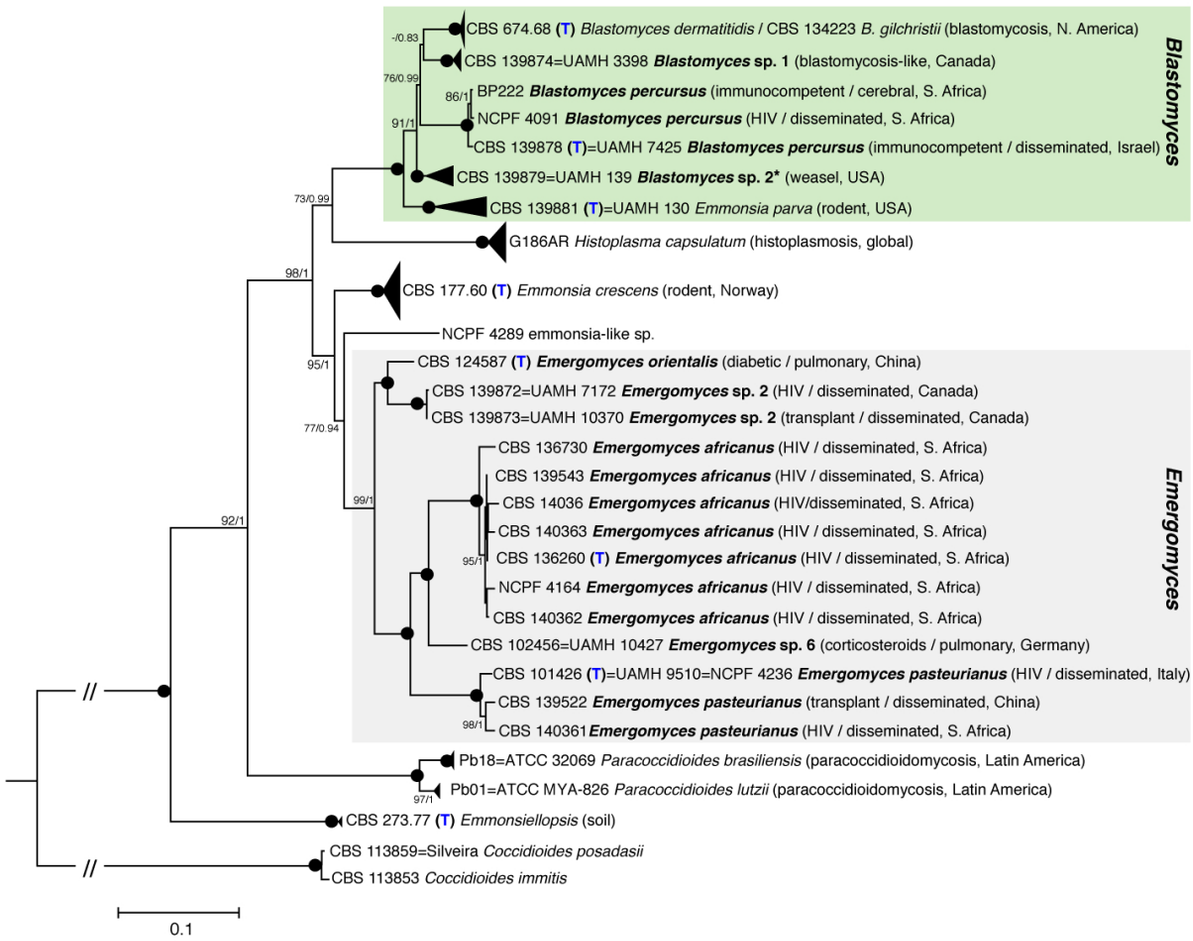

Figure 1. RAxML tree constructed from concatenated dataset including five regions: ITS, LSU, *TUB*, *TEF3* and *RP 60S L1*. The genera and species that are subject of this analysis are shown in bold font and represented by all analyzed strains (Table 1). Multiple sequences of other species/genera are collapsed (represented by one strain that is: type or neotype, genome sequenced or key strain from yet undescribed species). Bootstrap support (BS) from RAxML > 70% (left) and Bayesian posterior probability (PP) >0.80 are given at the nodes. Fully supported branches (100/1) are depicted as black circles. (*) Formerly *Emmonsia parva*. (T) – Type strain.

Onygenaceae) as outgroup, five monophyletic clades were clearly recognisable and highly supported within the family *Ajellomycetaceae*, representing the new genus, the systemic pathogens in the genera *Paracoccidioides, Histoplasma* and *Blastomyces,* and the recently established environmental genus *Emmonsiellopsis* [(35) Fig. 1]. Within the *Ajellomycetaceae*, the genus *Emmonsiellopsis* is located in an ancestral basal position, followed by *Paracoccidioides*. Bootstrap support (BS) and posterior probabilities (PP) of the genera *Emmonsiellopsis* and *Paracoccidioides* were both high (BS/PP 100/1).

Two clades (green and grey boxes; Fig. 1) were analysed in detail. The upper clade (green box) contains *Blastomyces* in its current sense and in total is interpreted to represent the genus *Blastomyces*. The *Blastomyces* clade has five monophyletic subgroups, all supported by highest BS/PP values of 100/1 (black dots). The uppermost clade contains the aetiological agents of blastomycosis in North America, *B. dermatitidis* and *B. gilchristii*, which could not be separated with the chosen set of loci. The closest clade to *B. dermatitidis/B. gilchristii* is indicated as *Blastomyces* sp. 1 and includes the strain CBS 139874 (=UAMH 3398) originating from Canada, which was reported as an unusual case of blastomycosis (36). The nearest clade to the group *B. dermatitidis/B. gilchristii*/*Blastomyces sp.* 1 (BS/PP 76/0.99) includes strains of a

novel species causing systemic mycosis in healthy human hosts, described here as *Blastomyces percursus*. Two of the three *B. percursus* strains, BP222 and NCPF 4091 originating from South Africa are grouped together (BS/PP 86/1). A third strain, CBS139878=UAMH 7425 originating from Israel, is slightly divergent. Two further clades (BS/PP 91/1, 100/1) include strains previously described as *Emmonsia parva*. The clade denoted as *Blastomyces* sp. 2 includes the genome-sequenced *Ea. parva* strain CBS 139879=UAMH 139 (6) isolated by W. L. Jellison from a weasel in the USA. This is clearly separated from the clade including another classical *Ea. parva* strain, CBS 139881 (=UAMH 130) isolated by C.W. Emmons, and confirms its basal position and the generic distinctions among *Ea. parva* strains noted previously (3). The Emmonsia-like strains producing small adiaspores phylogenetically cluster in *Blastomyces*, while *Ea. crescens* remains outside *Blastomyces* (Fig. 1).

The lower clade in Fig. 1 (grey box) is clearly separated from *Blastomyces* and *Histoplasma*, and has a large diversity of isolates, including emerging dimorphic species, and is here described as *Emergomyces*. The clade is monophyletic with BS/PP 99/1 support and includes three well-defined subclades. The monophyletic *Es. pasteurianus* cluster (BS/PP 100/1) comprises the type strain CBS 101426=UAMH 9510 and two additional strains. A second large monophyletic cluster (BS/PP 100/1) comprises seven strains obtained from HIV-infected individuals with disseminated mycoses in South Africa (9). The new taxon *Emergomyces africanus* has the genome-sequenced strain CBS 136260 as type. The group is separated from *Es. pasteurianus* by a single strain from a human infection in Germany, CBS 102456 (=UAMH 10427), which is significantly different from *Es. africanus* (e.g by 19/508 ITS alignment difference). Thus, it is denoted as *Emergomyces* sp. 6 [formerly *Emmonsia* sp. 6 in Schwartz et al. (1)]. A sister clade to the *Es. pasteurianus*-group comprises the recently described species *Es. orientalis* (7) and another undescribed taxon, *Emergomyces* sp. 2 [as *Emmonsia* sp. 2 in Schwartz et al. (1)] represented by two strains from Canada. All species of the *Emergomyces* clade produce small yeast-like cells when grown at 37 °C in vivo and in vitro, in contrast to the large yeast cells of *B. dermatitidis* and the large, thick-walled adiaspores of *Ea. crescens* which are difficult to obtain in culture.

Annotated genome assemblies of *Emergomyces pasteurianus*, *Es. africanus* and *Blastomyces percursus*

To better characterise the relationships of novel species and to provide reference genomes, we sequenced and assembled the genomes of the type or representative strains for each new species, *B. percursus* strain BP222, *Es. africanus* strain CBS 136260 and *Es. pasteurianus* strain CBS 101426. The species were sequenced using different technologies (Illumina MiSeq, IonTorrent and Illumina HiSeq 2000 respectively) and de novo assembled. The genome assembly sizes of these three species were 32.3 Mb in *B. percursus*, 29.7 Mb in *Es. africanus* and 32.4 Mb in *Es. pasteurianus* (Table 2, Fig. 2A). These assembly sizes are similar to those of other species within the *Ajellomycetaceae* including *Ea. parva*, *Ea. crescens*, *H. capsulatum* and *P. brasiliensis* (6, 29, 37) which have an average of 30 Mb. Further, the genomes of these species are not as expanded as are the genomes of the closely related species *B. dermatitidis* and *B. gilchristii*, which are 66.6 Mb and 75.4 Mb respectively (6). This includes the closely related strain BP222 representative of *B. percursus*, suggesting that the remarkable genome expansion in *B. dermatitidis* and *B. gilchristii* occurred recently.
The total number of predicted genes in *B. percursus*, *Es. africanus* and *Es. pasteurianus* was similar to that found in other *Ajellomycetaceae*, as well as in distantly related dimorphic fungal pathogens in the family *Onygenaceae*, such as *Coccidioides* (Fig. 2). Numbers of predicted genes for *B. percursus*, *Es. africanus* and *Es. pasteurianus* were 10 293, 8769 and 8950

Table 2. Assembly and annotation statistics for *Emergomyces pasteurianus*, *Es. africanus*, *Blastomyces percursus*. For comparison, GC-content %, genome size and number of genes were included for reference genomes of *B. dermatitidis* (ER-3), and *Blastomyces* sp. 2 (UAMH 139, formerly *Emmonsia parva*).

Genus	***Emergomyces***		***Blastomyces***		
Species	*Es. africanus*	*Es. pasteurianus*	*B. percursus*	*B. dermatitidis*	B. sp. 2
Strain	CBS136260	UAHM9510	BP222	ER-3	UAMH139
Scaffolds	4.444	1.643	3.868	25	2682
Scaffold N50 (kb)	13,8	45,9	12,8	5550	31,17
Scaffold N90 (kb)	2,5	8,8	4	2312	3,4
Max Scaffold (kb)	79,7	267,9	71,4	10.302,04	265,3
Assembly GC	43,5	44,5	47,3	37,1	44,7
Total Length (Mb)	29,7	32,4	32,3	66,6	30,3
Coding-protein genes	8.769	8.950	10.293	9.755	8.563

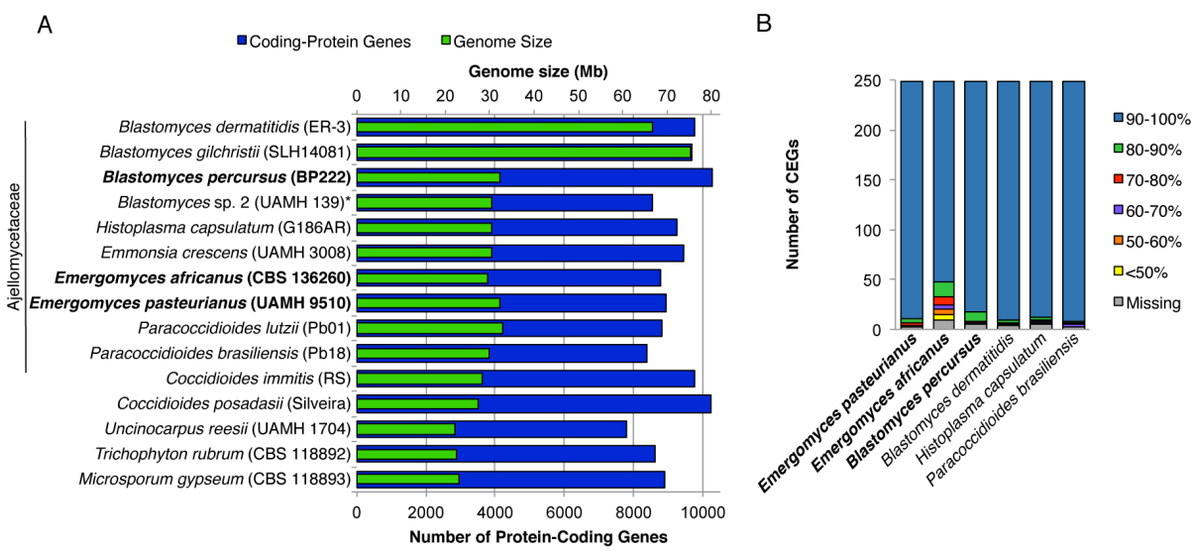

Figure 2. (A) Genome sizes in megabases (Mb) and total number of protein-coding genes of the sequenced and assembled strains of Emergomyces africanus, *Es. pasteurianus* and *Blastomyces percursus*, and other previously sequenced and assembled species from the *Ajellomycetaceae*. * formerly *Emmonsia parva* **(B)** Conservation of core eukaryotic genes (CEGs) across *Es. africanus*, *Es. pasteurianus*, *B. percursus* and other compared genomes.

respectively (Table 2, Fig. 2A). Despite the smaller contig size in two assemblies (Table 2), we found high representation of core eukaryotic genes in all genomes, providing evidence that their gene sets are nearly complete; *Es. africanus* included 88% of core eukaryotic genes, while *B. percursus* and *Es. pasteurianus* gene sets included 96–98% (Fig. 1B). Based on their completeness, the *B. percursus*, *Es. africanus* and *Es. pasteurianus* reference genome assemblies can define a wide set of genes that is shared across the dimorphic pathogenic fungi. We classified these references according to the mating type locus. *Es. africanus* and *Es. pasteurianus* contained mating-type alpha (*MAT1-1*; locus ID ACJ72_07256 and AI78_01298 respectively), while *B. percursus* contained the mating-type HMG (*MAT1-2*; locus ID ACJ73_00817).

Phylogenomics of *Emergomyces pasteurianus*, *Es. africanus* and *Blastomyces percursus*

To compare gene content and conservation, we identified orthologous gene clusters in the three genomes sequenced here, *Onygenales* genomes of other dimorphic pathogens (*Blastomyces*, *Histoplasma, Paracoccidioides* and *Coccidioides*) and two dimorphic non-human pathogenic species, *Ea. parva* and *Ea. crescens*, the aetiological agents of adiaspiromycosis in small mammals. As outgroups, three *Aspergillus* genomes were also included. Using 2851 single-copy core genes present in all strains, we estimated a strongly supported phylogeny of these organisms using RAxML (Fig. 3). *Blastomyces percursus* clustered with the primary pathogen *B. dermatitidis/B. gilchristii* (100% bootstrap support). *Blastomyces* sp. 2 (strain UAMH 139; formerly *Ea. parva*) was also closely related, branching earlier as a sister species within the *Blastomyces* clade (Fig. 3; green box). *Es. africanus* and *Es. pasteurianus* clustered in a single, strongly supported (100% bootstrap replicates) clade as sister species, and this clade was sister to *Ea. crescens* (Fig. 3; grey box). The *Ea. crescens – Emergomyces* clade is a sister group of the clade including *Histoplasma* and *Blastomyces*, with *Paracoccidioides* in a basal position.

Multilocus and genome analyses demonstrated that (i) one group of novel dimorphic species (*Emergomyces*; grey box) formed a single, derived clade within the *Ajellomycetaceae* distinct from the classic dimorphic pathogens and classic *Emmonsia* species; (ii) a second group (*B. percursus*; within green box) clustered as a separate clade within the genus *Blastomyces* as close relative to the dimorphic pathogens *B. dermatitidis/B. gilchristii* and (iii) the aetiological agents of adiaspiromycosis (*Ea. parva* and *Ea. crescens*) in the current sense are polyphyletic with *Ea. parva* strains having small adiaspores grouping with *Blastomyces* and *Ea. crescens* having large adiaspores occupying a unique position.

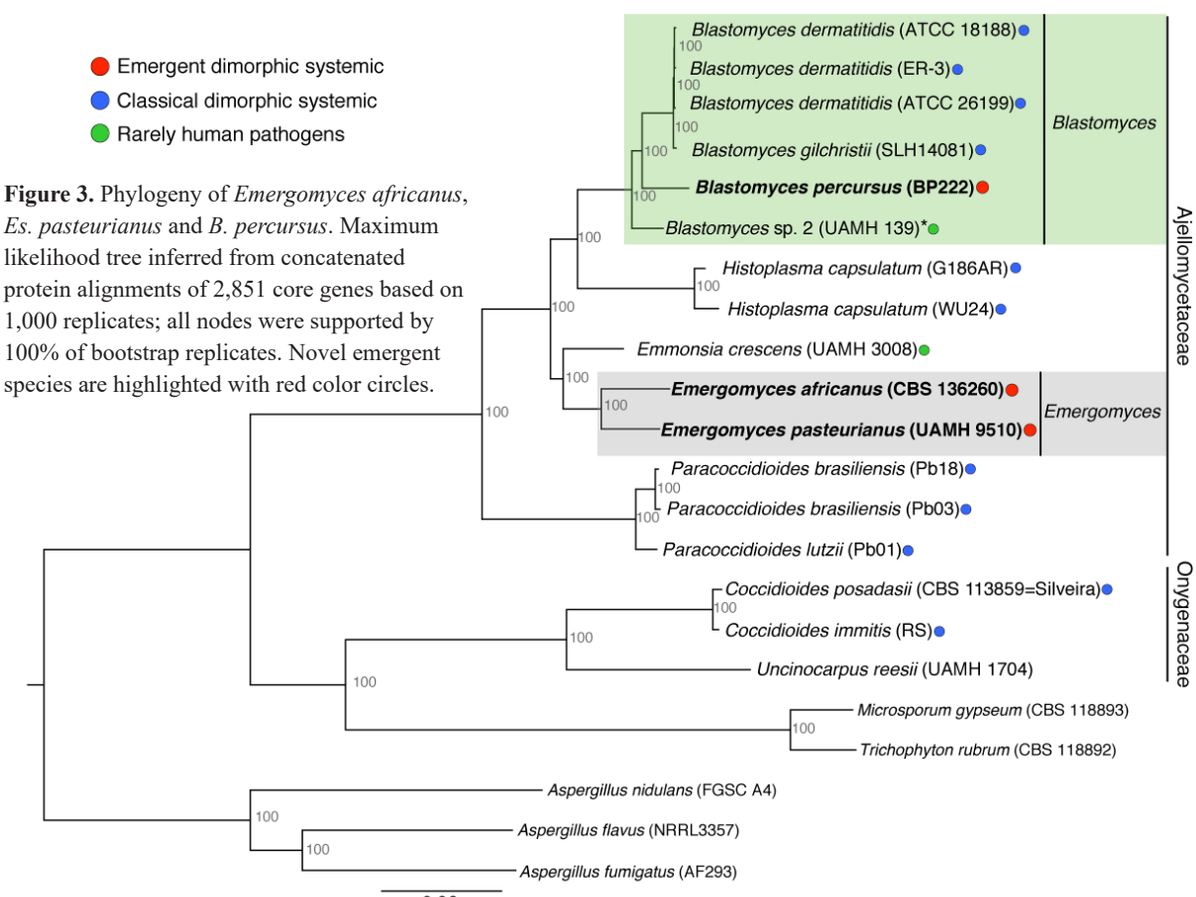

Figure 3. Phylogeny of *Emergomyces africanus*, *Es. pasteurianus* and *B. percursus*. Maximum likelihood tree inferred from concatenated protein alignments of 2,851 core genes based on 1,000 replicates; all nodes were supported by 100% of bootstrap replicates. Novel emergent species are highlighted with red color circles.

Members of the *Emergomyces* clade produce filamentous phases similar to those of other members of the *Ajellomycetaceae*, that is, solitary, single-celled conidia being formed on short narrow pedicels on swollen conidiogenous cells at 24 °C (Figs. 4 and 5). Their responses at 37 °C generate the most significant differences from the *Blastomyces* clade. Species of the *Emergomyces* clade (grey boxes in Figs. 1 and 3) produce small budding yeast cells (<5 µm) with a narrow base; budding in *Es. pasteurianus* may be uni-, bior multipolar. In contrast, members of the *Blastomyces* clade (green boxes in Figs. 1 and 3) typically produce larger yeast cells (>5 µm) with budding at a broad base in the case of *B. dermatitidis, B. gilchristii* and *B. percursus*.

Emergomyces Dukik, Sigler & de Hoog, **gen.nov**. – MycoBank MB818569
Etymology: referring to the newly emerged group of species during the last few decades.
Colonies on MEA at room temperature, white to beige, slow growing, filamentous. Hyphae hyaline, thin-walled. Conidiophores are short, unbranched, arising at right angles from hyphae, slightly swollen at the top, sometimes with short, secondary conidiophores. Conidia solitary, one-celled, often subspherical, produced on narrow pedicels on swollen conidiophores or sessile, or alongside hyphae. At 37°C, small yeast cells having narrow buds typically formed but larger cells with broader based buds sometimes present.
Type species: Emmonsia pasteuriana Drouhet et al. (10).
Differential diagnosis: The genus is distinguished from *Emmonsia* and *Blastomyces* species by small yeast cells budding at narrow bases at 37°C. It is clearly segregated from remaining genera of *Ajellomycetaceae* by multilocus sequence data. Strains attributed to the genus have been isolated from humans and cause primarily disseminated disease frequently characterised by cutaneous lesions.

Emergomyces pasteurianus (Drouhet, Guého & Gori) Dukik, Sigler & de Hoog, **comb. nov.** – MycoBank MB818570
Basionym: Emmonsia pasteuriana Drouhet, Guého & Gori, *in* Drouhet, Guého, Gori, Huerre, Provost, Borgers & Dupont – *J. Mycol. Méd.* 8: 90, 1998 (10).
Colonies at 24 °C on MEA whitish, composed of hyaline hyphae.
Conidiophores short, slender, unbranched, arising at right angles from narrow hyphae and slightly swollen at the tip; bearing 1-3 (up to 8) conidia on short thin pedicels or sometimes sessile alongside hyphae. Conidia subhyaline, slightly verruculose, thin-walled, onecelled, subspherical, 2-3 × 3-4 µm. At 37 °C, budding cells present which are ellipsoidal, 2-4 µm in length, budding at a narrow base, in addition to broad-based budding cells.
Physiology: minimum growth temperature 6 °C, optimum 24 °C, reaching 35 mm diameter, maximum 40 °C.
Type: CBS 101426=UAMH 9510=IP 2310.95=NCPF 4236, isolated from cutaneous lesion of 40-year-old female with advanced HIV disease and a history of injection drug use with disseminated fungal infection, Italy, 1984, reported in 1998 (25).
Differential diagnosis: This species is characterised by formation of small, ellipsoidal yeast-like cells (2-4 µm) at 37 °C, showing narrow-based, mostly unipolar budding, with rare bi or multipolar scars, intermingled with broad-based budding cells. In vivo, along with small thin-walled yeast cells, few larger thick-walled yeast cells
of 8-10 µm were observed(10).

Emergomyces africanus Dukik, Kenyon, Govender, Schwartz & de Hoog, **sp. nov.** – Figure 4, MycoBank MB818571
= *Emmonsia* sp., Kenyon et al. – *N. Engl. J. Med.* 369: 1416. 2013 (9).

= *Emmonsia* sp. 5, Schwartz et al. – *PLoS Pathog*. 11: e1005198 p. 2. 2015 (1).
Etymology: referring to the species causing outbreaks in South Africa.
Holotype: New Somerset Hospital, Cape Town, South Africa, specimen of culture CBS 136260 (preserved in metabolically inactive condition in liquid nitrogen) from skin biopsy of an HIV-infected male, collected by N.P. Govender, 11 June 2010.
Colonies on MEA at 24 °C, 4 week moist, circular, flat or slightly raised towards the centre, reaching 21 mm diameter, often with central hyphal tufts but otherwise lacking aerial mycelium. The firm hyphal mat is almost concolorous with agar. Colony reverse warm-buff in the centre, light buff around, radially sulcate. Mycelium delicate, hyphae 1.4–2.5 µm in diameter, hyaline, septate, branched, with few spirally twisted hyphae. Conidiophores mostly one-celled, solitary, arising at right angles from vegetative hyphae, 0.6–1.5 µm in diameter, with a septum at the base and mostly swollen at the tip; usually forming short, secondary conidiophores. Conidia emerging from swollen tips on narrow pedicels, each forming a terminal conidium, establishing a grouping or 'floret' of four to eight conidia. Conidia solitary, occasionally in chains of two or four, subspherical, slightly shortened along the vertical axis, 1.2-3.2 × 1.7-3.8 µm (2.2±0.5 × 2.7±0.5, n=45), smooth-walled to finely roughened; rhexolytic, sometimes adherent to the conidiophore; rarely sessile. Colonies on MEA at 37°C, 4 week smooth, glistening, cream-coloured to greyish-brown, reaching 5 mm diameter. Yeast cells abundant, ovoidal to subspherical, 1.7-5.3 × 0.9-2.2 µm (2.9±0.73 × 1.6±0.31, n=45), mostly single, occasionally multiple. Budding unilateral from a narrow base. Some swollen and short hyphae also present.
Physiology: minimum growth temperature 6 °C, optimum 24-27 °C reaching 21 mm diameter, maximum 40 °C.
Differential diagnosis: *Es. africanus* is differentiated by having small, ovoidal to subspherical yeast cells below 5 × 3 µm at 37 °C, budding at narrow bases at the poles. At 24 °C, conidia are borne in a complex cluster or 'floret' of four to eight conidia formed individually at the ends of slender stalks.

Blastomyces percursus Dukik, Muñoz, Sigler & de Hoog, **sp. nov.** – Figure 5, MycoBank MB 817662 = *Emmonsia* sp. 3, Schwartz et al. – *PLoS Pathog*. 11: e1005198 p. 2. 2015.
Etymology: referring to the ability of the fungus to infect multiple sites of human patients.
Holotype: Israel, specimen of culture CBS 139878 (preserved in metabolically inactive condition in liquid nitrogen) from granulomatous lesion on lip of otherwise healthy patient with disseminated infection, isolated by I. Polachek, November 1993; living strain CBS
139878=Kemna 408-93=UAMH 7425=UAMH 7426.
Colonies on MEA 24 °C, 4 week flat, reaching 42-43 mm diameter, with a loose, whitish felt of aerial mycelium and often with central hyphal tufts. Margin flat, with reptant hyphae. Colony reverse pale buff, warm-buff at the centre, radially sulcate from the centre. Exudate absent. Hyphae 1.1-2.8 µm in diameter, hyaline, septate, irregularly branched, locally swollen, with some spirally twisted hyphae. Conidiophores solitary, arising at right angles from vegetative hyphae, mostly swollen around the middle or near the end, 1.6-4.1 (2.2±0.59) µm wide, with a septum at the base and directly below the conidium, sometimes bearing 2-4 secondary conidiophores; solitary conidia produced on short and narrow pedicels of <1 µm long. Conidia solitary, subspherical, 1.5-4.4 × 1.7-4.6 µm (2.7±0.6 × 2.6±0.5, n=45), smooth to slightly roughened, rhexolytic, sometimes adherent to the conidiophore. Chlamydospore-like cells occasionally present on short lateral branches, having thickened cell walls and often a median septum.

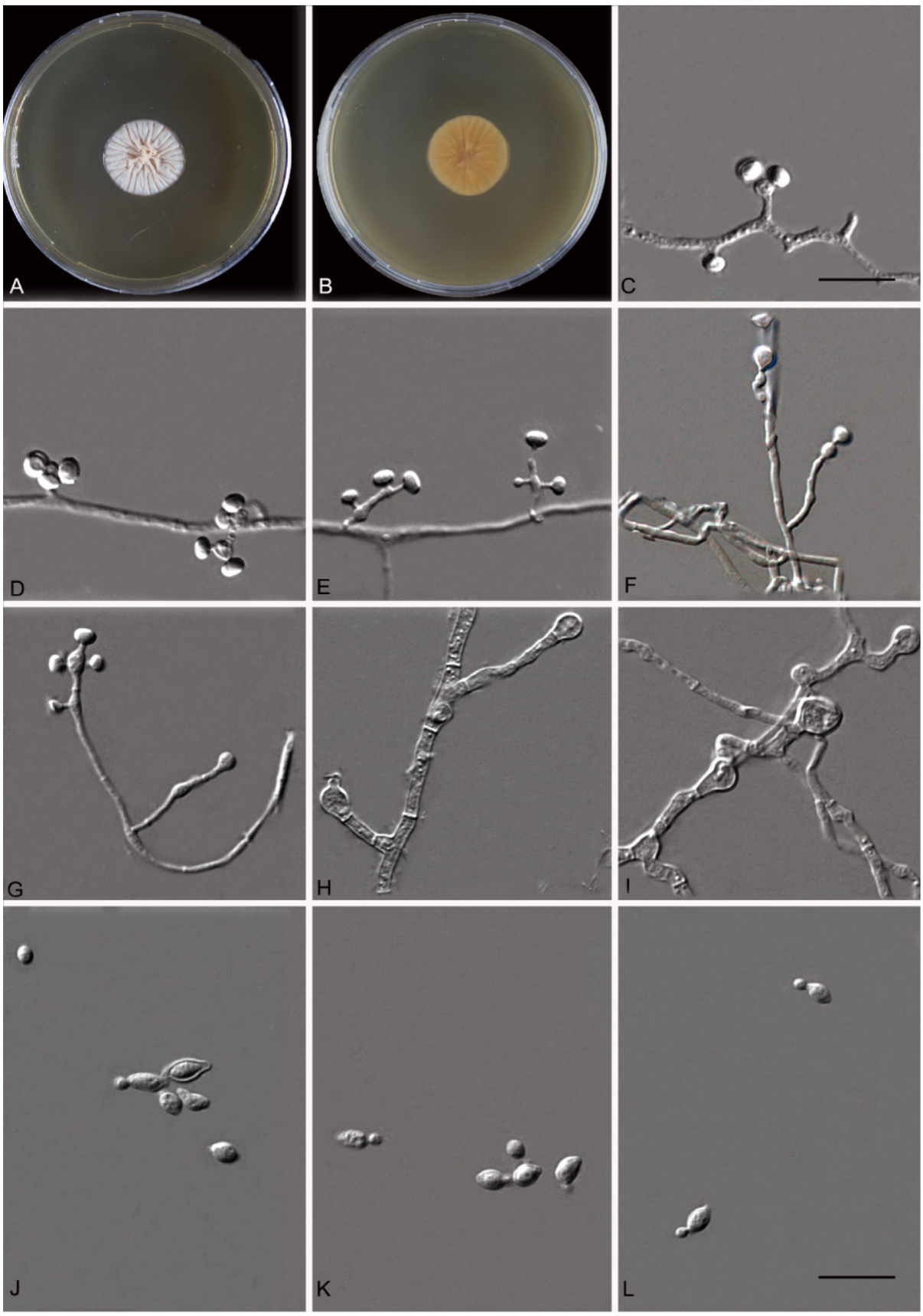

Figure 4. *Emergomyces africanus* sp. nov., CBS 126360 (type strain). (A, B) Colony on MEA after 4 weeks at 24 °C, obverse and reverse. (C−I) Conidia of saprophytic phase. (J−L) Small yeast of parasitic phase at 37 °C. Scale bars = 10 µm.

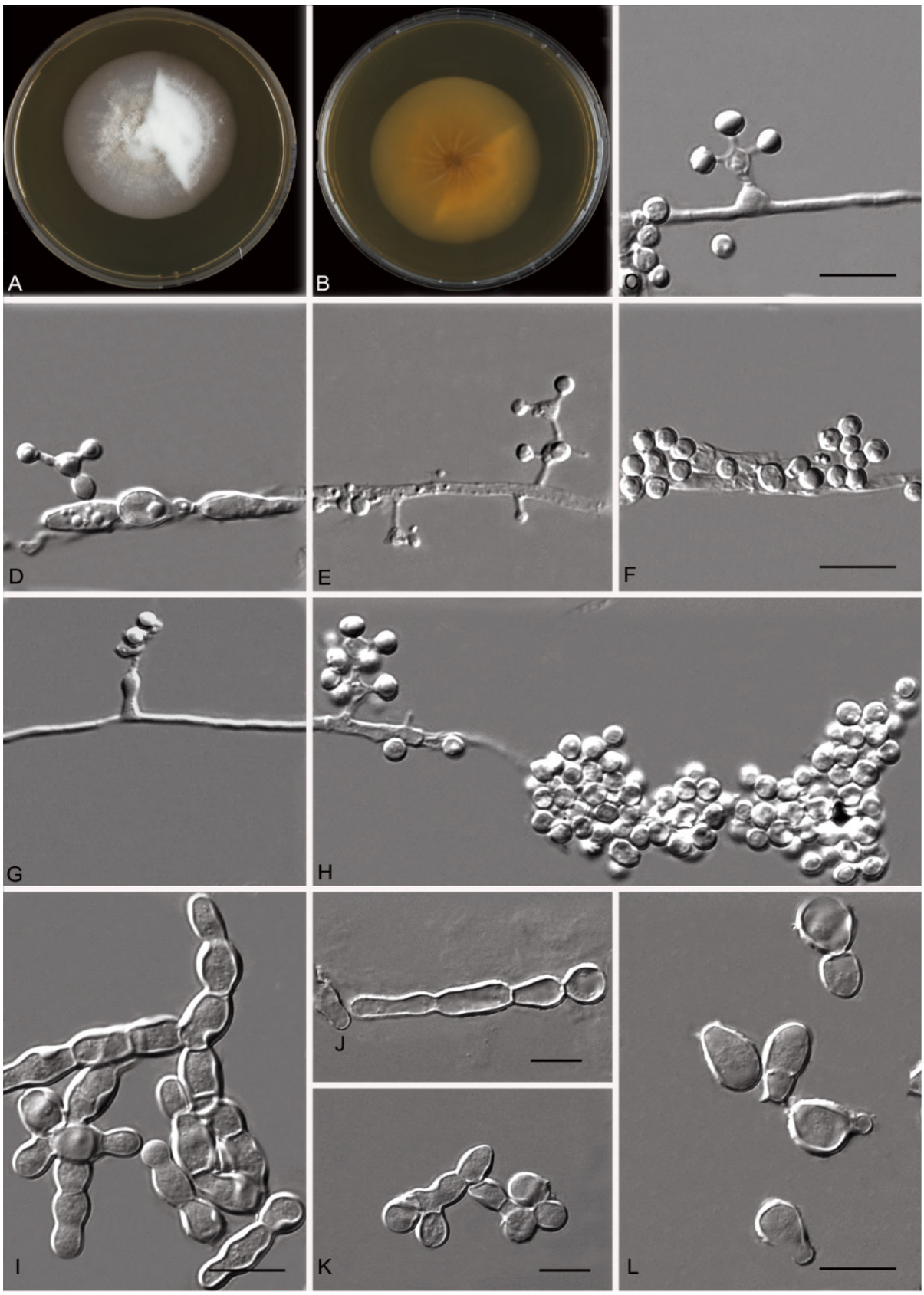

Figure 5. *Blastomyces percursus* sp. nov., CBS 139878 (type strain). (A, B) Colony on MEA after 4 weeks at 24 °C, obverse and reverse. (C−H) Conidia of saprophytic phase. (I−L) Large yeast with unipolar or bipolar budding at a broad base at parasitic phase at 37 °C. Scale bars = 10 µm.

Colonies on MEA at 37 °C, 4 week smooth, shiny, cream to greyish-brown, reaching about 6 mm diameter. Commonly short and swollen hyphal elements present with shorter intercalary cells and disarticulating into smaller fragments, intermingled with large yeast-like cells with unior bipolar budding at a broad base, 5.2-12.2 × 2.4-6.5 µm (8.1±1.7 × 4.8±0.90, n=45).
Physiology: minimum growth temperature 9-15 °C, optimum 27 °C reaching 48 mm, maximum 40 °C.
Differential diagnosis: This species differs in having more elaborate conidiophores bearing conidia on stalks, in contrast to the simple conidiogenous cells bearing single conidia that are typical of *B. dermatitidis/B. gilchristii*. Yeast cells produced at 37 °C are subspherical, over 5 µm long, and bud at a broad base as in *B. dermatitidis*.

Discussion

Recent reports document the emergence of infections in humans caused by new types of systemic thermo-dimorphic fungi over the past few decades (1, 7, 9, 10, 12, 24, 25, 38-40, 47). Initially, these fungi were considered Emmonsia-like, based on the microscopic morphology of conidia, which cluster in florets on conidiophores (1). However, their yeast-like appearance in tissue and in culture at 37 °C differed from classical species of *Emmonsia*. The disease caused by *Emmonsia* species is adiaspiromycosis, a pulmonary disease occurring in rodents and other small burrowing animals in which the fungus resides as spherical structures in the lungs, known as adiaspores which enlarge but do not multiply, in contrast to the endosporulating spherules of *Coccidioides* species (5). Human infections are uncommon (4, 5). The discovery of adiaspiromycosis occurred in 1942 when Emmons and Ashburn (41) observed a fungus producing spherule-like structures up to 20 µm in the lungs of rodents trapped in Arizona, U.S.A. The fungus was described originally as *Haplosporangium parvum*, but was reclassified as the type species of a new genus *Emmonsia* as *Emmonsia parva*. The second described species, *Ea. crescens* differed in producing giant adiaspores in tissue up to 200 µm in diameter, which could be reproduced in vitro at 37 °C (5, 42). Peterson and Sigler (3) found that *Ea. parva*, the type species of *Emmonsia,* is phylogenetically closely related to *B. dermatitidis*. This was confirmed in our multilocus and phylogenomic data. With the type species of the genus *Emmonsia* clustering in *Blastomyces*, the former genus becomes a synonym of *Blastomyces*. Peterson and Sigler (3) showed *Ea. crescens* to belong to a single, rather invariable clade and our data indicate that the species is separated from *Blastomyces* by *Histoplasma*. Its exact phylogenetic position and its relationship with other taxa within the *Ajellomycetaceae* remain unresolved. *Ea. crescens* is one of only three taxa within the *Ajellomycetaceae* including *B. dermatitidis* and *H. capsulatum* that are heterothallic and have proven Ajellomyces-like sexual stages (43). The first human-associated species classified in *Emmonsia* was *Ea. pasteuriana* (10) but it was fundamentally different, particularly from *Ea. crescens* in the type of infection caused (disseminated cutaneous mycosis rather than limited pulmonary disease), in production of yeast-like cells rather than adiaspores in tissue and in its occurrence in humans. *Emergomyces pasteurianus* was first isolated in 1994 from an Italian woman with advanced HIV-infection presenting with disseminated skin lesions in which small budding yeast cells of 2-4 µm were observed in tissue and in culture at 37 °C (10, 25). Additional reports can be attributed to *Es. pasteurianus.* A case of disseminated infection occurred in Spain in an HIV-infected person who was also a liver transplant recipient and presented with pulmonary and skin lesions (44). Malik et al. (11) reported an *Es. pasteurianus* disseminated infection in an Indian woman with advanced HIV who presented with multiple non-

tender skin lesions and pulmonary disease. Two further cases involved disseminated skin infection in non-HIV-infected patients from Guangzhou, China; one occurred in a renal transplant recipient (45) and another in a male receiving high dose corticosteroids (46). Additionally, we report that *Es. pasteurianus* has been isolated from a patient in South Africa (Fig. 1, Table 1). The clinical, morphological and phylogenetic analyses described here determined that *Ea. pasteuriana* and several other Emmonsia-like fungi with yeast stages formed a single, derived clade in the *Ajellomycetaceae*, described here as *Emergomyces* with type species *Es. pasteurianus*.

The emergence of *Es. africanus* as the cause of disease among HIV-infected persons from South Africa and the Kingdom of Lesotho has been remarkable with respect to the numbers of cases reported (1, 8, 9, 39). Thirteen cases were discovered during an initial surveillance programme in South Africa using broad-range fungal PCR assay of all deep fungal infection clinical isolates (9); the number of reported cases was soon expanded to 52 (8). Fifty-one patients had advanced HIV disease, and one was a renal transplant recipient. Ninety-five per cent of patients had widespread skin lesions, which were protean and often misdiagnosed. Isolates were primarily cultured from skin and bone marrow biopsies or blood culture. Another species, *Es. orientalis* has been described separately for a strain from Beijing, China causing disseminated infection in an individual with diabetes (7). Other fungi appear to warrant placement in *Emergomyces*. These include a strain recovered from the lung tissue of a male with rheumatoid arthritis treated with low doses of corticosteroids in Germany (38) (Fig. 1 as *Emergomyces* sp. 6). Molecular analysis of two isolates from immunocompromised patients in Canada (40) placed them as sister clade to *Es. orientalis* (Fig. 1 as *Emergomyces* sp. 2). Taken together, these cases underline the potential of *Emergomyces* species as new cosmopolitan opportunistic pathogens in the immunocompromised host. Most persons infected with *Emergomyces* species have impaired cellular immunity. Some cases now attributed to *Es. africanus* infection were misclassified because they were incorrectly diagnosed by histopathological examination as *H. capsulatum* (1, 9). Based on the sequencing data, none of the *Histoplasma* strains retained in the CBS collection represents any of the novel dimorphic fungi described here. The environmental reservoir is unproven although early evidence has implicated soil (I. Schwartz, unpublished data). Infection of animals has not been reported.

Strains identified as *Blastomyces percursus* came from immunocompetent and immunocompromised hosts. The type strain (CBS 139878=UAMH 7425) was found to cause granulomatous oral lesions in an immunocompetent patient in Israel (47). Molecular analyses by Peterson and Sigler (3) and Schwartz et al. (1) placed this isolate in the *Blastomyces* clade. Our analysis of this strain and two additional isolates confirmed this, and showed a clear separation between this clade as compared with *B. dermatitidis/B. gilchristii*. Two other strains are from cases in South Africa (9, 24). The first case was isolated from ulcerated skin of an HIV-infected person in Johannesburg in 1986 and it was originally identified as *B. dermatitidis* (NCPF 4091). The second strain (BP222) came from a 52-year-old previously healthy male with a cerebellar abscess. The diagnosis was based on a brain tissue biopsy which showed budding yeast cells suggestive of *B. dermatitidis*. However, sequencing of the ITS locus showed partial alignment with the ITS sequences of the South African Emmonsia-like strains. The patient received amphotericin B followed by oral itraconazole therapy with good clinical response.

The polyphyletic nature of the analysed dimorphic human pathogens and the aetiological agents of adiaspiromycosis (*Ea. parva* and *Ea. crescens*) which are separated from each other by species with other types of pathogenic phases suggest that members of the *Ajellomycetaceae* have undergone multiple evolutionary transitions allowing infection

of humans and other mammals. In addition, it shows how, in spite of phylogenetic transitions, they have retained their mesophilic morphology including sporulation with solitary, slightly to moderately rough-walled conidia; *Histoplasma* is exceptional in its production of conidia of two sizes, the larger being coarsely ornamented (tuberculate). Major differences between species and genera (*Blastomyces, Emergomyces, Histoplasma, Paracoccidioides*) are in their invasive forms, whereby *Histoplasma* is again exceptional in its intracellular growth of small yeast cells in host macrophages. It may be noted that the biological coherence of taxa in *Ajellomycetaceae* is not only underlined by monomorphic filamentous stages occurring throughout the family, but also by *Ajellomyces* teleomorphs having elaborate morphology of gymnothecia, asci and ascospores. From a point of view of ambient morphology at room temperature, sexual as well as clonal, all members of *Ajellomycetaceae* show a number of highly conserved traits, which likely are linked to their alternating life cycle with mammal hosts.

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Conflict of interests

None to declare.

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CHAPTER 6

Antifungal susceptibility of emerging dimorphic pathogens in the family *Ajellomycetaceae*

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Abstract

The *in vitro* susceptibility of 24 molecularly identified dimorphic fungi belonging to *Adiaspiromyces, Blastomyces* and *Emergomyces* within the family *Ajellomycetaceae* was tested against 8 standard antifungals using CLSI M38-A2. Amphotericin B and posaconazole had the lowest geometric mean MICs (< 0.05µg/ml) followed by itraconazole (< 0.07µg/ml), voriconazole (< 0.15µg/ml) and isavuconazole (< 0.42µg/ml) while fluconazole was not active. Micafungin demonstrated good in vitro antifungal activity against *Emergomyces* (GM-MEC 0.1µg/ml) and *Blastomyces* (GM-MEC < 0.017µg/ml).

Keywords

Dimorphic fungi, *Adiaspiromyces, Blastomyces*, *Emergomyces,* antifungal susceptibility testing, CLSI

Introduction

Fungi in the family *Ajellomycetaceae* are well known systemic dimorphic pathogens. Recent phylogenetic studies resulted in establishment of two new genera (*Adiaspiromyces* and *Emergomyces*) in addition to the well-known genera *Blastomyces, Histoplasma* and *Paracoccidioides* (1-3). The genus *Adiaspiromyces* is now accommodating the former *Emmonsia crescens* and the environmental species *Adiaspiromyces terricola* (2). The genus *Emergomyces* contains *Emergomyces pasteurianus* as a type species *(*formerly *Emmonsia pasteuriana*) and four newly introduced species, *Emergomyces africanus* (1), *Emergomyces orientalis* (4), *Emergomyces canadensis* and *Emergomyces europaeus* (2). *Blastomyces* was reorganized to include three new species, *B. silverae*, *B. helicus and B. percursus* along with *Blastomyces parvus* (former *Emmonsia parva*), *B. gilchristii* and the well-known human pathogen *B. dermatitidis* (1, 2).

These genera and their species differ in their pathogenic forms (adiaspores, small - and large yeasts), host range and clinical manifestation. *Adiaspiromyces crescens* is primarily a causative agent of pulmonary infections in terrestrial mammals (5) but has been reported occasionally as a causative agent of pulmonary infections in humans (6). The pathogenic form is called adiaspore, a large spore with dimensions ranging from 20-140 µm (7). Since human infections are rare, there are only few reports on antifungal susceptibility testing (5) or treatment (6).

On the contrary, *Emergomyces* species are human pathogens and not yet isolated from the environment or from animals. Almost all infected human hosts were HIV infected. *Emergomyces* species differ from the other species within the same family by producing budding yeasts (less than 5 µm) *in vivo* rather than adiaspores and often disseminate with secondary cutaneous manifestation, referred to as emergomycosis (8). Two species have been tested for antifungal susceptibility: *Emergomyces africanus,* the causative agent of an outbreak in South Africa (8, 9) and *Emergomyces pasteurianus* (10). *Emergomyces* infections have been treated mostly with amphotericin B, itraconazole or fluconazole (4, 11, 12) but no clinical trial has been conducted to date.

The genus *Blastomyces* is more versatile than *Adiaspiromyces* and *Emergomyces*. *Blastomyces dermatitidis*, *B. gilchristii* and *B. percursus* are human pathogens with broad-based large yeasts (more than 5µm) as pathogenic form (2). *Blastomyces dermatitidis* and *B. gilchristii* cause blastomycosis mostly in North America (13) with few cases reported from the Asia-Pacific region (14,15). There are several antifungal susceptibility studies (16-18) and treatment suggestions (19,20). *Blastomyces percursus* was recently described as a human pathogen in both immunocompetent and immunocompromised patients (21). The other two species, *B. parvus* (*Emmonsia parva*) and the newly described *B. silverae* cause infections in small animals but rarely in humans (2). No in vitro susceptibility data of these two species have been published.

We report *in vitro* antifungal susceptibility of eight antifungals of the newly described species *Emergomyces africanus*, *E. orientalis*, *E. canadensis*, *Blastomyces percursus*, *B. helicus* and *B. silverae*. Although *in vitro* antifungal susceptibility testing of these dimorphic fungi is not standardized, the obtained MIC data might help with treatment decisions for infections caused by these fungi. Of twenty-four isolates, 14 (58.3%) originated from human patients, four (16.6%) from rodents, two (8.3%) from soil, and one (4.16%) each from a weasel, from coyote dung, from bird's nest and of unknown origin. It should be noted that these species are still rare, and thus only

small numbers of strains per species are available: *Emergomyces pasteurianus* (n=3)*, E. africanus* (n=5), *E. orientalis* (n=1)*, E. canadensis* (n=2), *Adiaspiromyces crescens* (n=4), *B. gilchristii* (n=1), *B. parvus* (n=2)*, B. helicus* (n=1)*, B. silverae* (n=3) and *B. percursus* (n=1), with one strain of *Blastomyces dermatitidis* as reference. All strains were initially subcultured on malt extract agar (MEA, Oxoid, UK) and molecularly identified using multilocus sequencing as previously described (1).

Materials and methods

Susceptibility studies were conducted in a class II biosafety cabinet in BSL 2 laboratories, except for *B. dermatitidis* and *B. gilchristii* which were tested using a BSL 3 facility. The isolates were subcultured on potato dextrose agar (PDA, Oxoid, UK), malt extract agar (MEA, Oxoid, UK) and Sabouraud's glucose agar (SGA). Sporulation on PDA was most abundant and up to ten plates with this medium were used to collect sufficient amounts of conidia. Antifungal susceptibility testing (AFST) was determined by broth microdilution using CLSI document M38A2 (22). The mold phase on PDA at 24 °C was used for inoculum preparation, which was adjusted to an optical density at 530 nm, to present transmission in a range of 76–77 % corresponding to 2.5–5 10^5 CFU per ml and diluted 1:10 in RPMI 1640 medium. Plates were incubated at 24 °C for 6 days with the following drug concentration ranges of amphotericin B (Bristol Myers Squib, Woerden, The Netherlands), itraconazole (Janssen Cilag, Beerse, Belgium), voriconazole (Pfizer Central Research, Sandwich, U.K.), posaconazole (Merck, Sharp & Dome, Haarlem, The Netherlands), and isavuconazole (Basilea Pharmaceuticals, Basel) of 0.016–16 µg/ml, fluconazole (Pfizer) of 0.063–64 µg/ml and anidulafungin (Pfizer) and micafungin (Astellas Pharma, Toyama, Japan) 0.008–8 µg/ml. The quality of every new batch of MIC plates was tested by using two QC stains, *Candida parapsilosis* ATCC 22016 and *Aspergillus flavus* ATCC 204304.

Results

Table 1 summarizes the MICs of 24 isolates from three clinically related genera, *Adiaspiromyces*, *Emergomyces* and *Blastomyces.* All *Emergomyces* species had MIC values of ≥64 µg/ml for fluconazole, except *Emergomyces canadensis* which had a MIC of 8 µg/ml. *Adiaspiromyces* species showed high MICs of 16 µg/ml, with one isolate having the highest value of 64 µg/ml. *Blastomyces silverae* showed the highest MIC with ≥64 µg/ml, followed by *B. parvus* and *B. helicus* with 32 µg/ml respectively. *Blastomyces dermatitidis*, *B. gilchristii* and *B. percursus* had the lowest MICs (2 µg/ml) of all species. For the other azoles, a low degree of variation was found at the generic level. Overall, all MIC values of azole compounds except fluconazole were low. Amphotericin B was the most active antifungal agent and had the lowest MICs against species of *Adiaspiromyces*, *Emergomyces* and *Blastomyces.* At the genus level, GM MIC and MIC ranges of amphotericin B were 0.027 and <0.016–0.125 µg/ml for *Adiaspiromyces*, 0.049 and <0.016–0.025 µg/ml for *Emergomyces* and 0.023 and <0.016–0.063 µg/ml for *Blastomyces*. GM MIC values of itraconazole, voriconazole, posaconazole and isavuconazole were comparable to the values of amphotericin B (Table 2).
Anidulafungin had low GM MEC and MEC ranges of 0.68 and 0.031-2 µg/ml and 0.18 and 0.016-1 µg/ml for the genera *Emergomyces* and *Blastomyces* respectively. Micafungin showed consistent low values with GM MEC and MEC ranges of 0.1 and 0.016-1 µg/ml and 0.017 and 0.016-0.063 µg/ml for *Blastomyces* and *Emergomyces* respectively.

Table 1. In vitro susceptibility of 24 *Adiaspiromyces, Blastomyces* and *Emergomyces* strains.

No	Species name	CBS no.	MIC (mg/L)								Source	Origin
			AMB	FLC	ITC	VOR	POS	ISA	ANID	MICA		
1.	*Emergomyces pasteurianus*	101426 (T)	0,031	>64	0,125	0,25	0,125	2	0,5	0,031	human, HIV, disseminated	Italy
2.		140361	0,063	64	0,063	0,25	0,063	0,5	0,5	0,016	human, HIV, disseminated	S.Africa
3.		139522	0,125	>64	0,25	0,25	0,063	1	0,031	0,063	human, renal transplant, disseminated	China
4.	*Emergomyces africanus*	140362	0,25	64	0,125	0,063	<0.016	0,125	1	1	human, HIV, disseminated	S.Africa
5.		136260 (T)	0,031	64	0,063	0,125	0,063	0,5	2	0,031	human, HIV, disseminated	S.Africa
6.		4164	0,063	64	0,063	0,063	0,031	0,125	2	1	human, HIV, disseminated	S.Africa
7.		140363	0,031	64	<0.016	0,063	0,031	0,25	1	0,063	human, HIV, disseminated	S.Africa
8.		139543	0,031	64	0,063	0,25	0,031	2	0,5	0,125	human, HIV, disseminated	S.Africa
9.	*Emergomyces orientalis*	124587	0,063	>64	0,031	0,125	0,031	0,25	1	0,125	human, diabetes, disseminated	China
10.	*Emergomyces canadiensis*	139873	0,031	8	0,063	0,125	0,063	0,25	0,5	0,063	human, transplant, disseminated	Canada
11.		139872 (T)	<0.016	>64	0,031	0,125	0,031	0,25	2	0,25	human, HIV, skin lesion	Canada
12.	*Adiaspiromyces crescens*	139864	<0.016	16	0,031	0,063	<0.016	0,125	2	0,125	rodent	Korea
13.		191,55	<0.016	16	<0.016	<0.016	<0.016	<0.016	4	0,063	rodent	Canada
14.		177.60 (T)	<0.016	16	0,031	0,063	0,031	0,125	8	8	rodent	Norway
15.		139869	0,125	16	0,031	0,125	0,063	0,25	8	0,125	environmental	Canada
16.	*Blastomyces dermatitidis*	126,33	0,016	2	<0.016	0,063	0,016	0,063	0,031	0,008	no data	no data
17.	*Blastomyces gilchristii*	134223	0,031	2	0,031	0,063	0,031	0,125	0,016	0,008	human	Canada
18.	*Blastomyces parvus*	139881(T)	<0.016	32	0,016	0,125	0,031	0,25	0,5	0,031	rodent	USA
19.		139883	<0.016	32	<0.016	0,063	0,031	0,25	0,25	0,031	soil	Italy
20.	*Blastomyces helicus*	140056(T)	<0.016	32	<0.016	<0.016	<0.016	<0.016	0,063	0,0031	human, leukemia	Canada
21.	*Blastomyces silveriae*	139879(T)	<0.016	64	0,125	0,125	0,125	0,25	1	0,063	weasel	USA
22.		139885	0,063	64	0,031	0,063	0,063	0,25	1	0,016	coyote dung	Canada
23.		139871	0,063	>64	0,125	0,125	0,063	0,5	0,25	0,031	bird's nest	Canada
24.	*Blastomyces percursus*	139878	<0.016	2	<0.016	0,031	0,031	0,031	0,25	0,031	Human. immunocompetent	Israel

MIC/MEC values determined according to CLSI M38-A2; MIC = minimum inhibitory concentration; MEC = minimum effective concentration; AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; VOR, voriconazole; POS, posaconazole; ISA, isavuconazole; ANID, anidulafungin; MICA, micafungin.

Table 2. MIC/MEC range, GM and MIC/MEC50-90 values for *Adiaspiromyces*, *Emergomyces* and *Blastomyces* to eight antifungal drugs.

MIC/MEC [µg/ml; range GM]								
Genus	**AMB**	**FLC**	**ITC**	**VOR**	**POS**	**ISA**	**ANID**	**MICA**
Adiaspiromyces (n=44) GM MIC/MEC	<0.016-0.125 0.027	16-64 22.6	<0.016-0.031 0.026	<0.016-0.125 0.05	0.016-0.063 0.026	<0.016-0.125 0.088	2- 8 4.75	0.125-8 0.29
Emergomyces (n=11) GM MIC/MEC MIC/MEC50 MIC/MEC90	<0.016-0.25 0.049 0.031 0.125	8->64 46.7 64 64	<0.016-0.25 0.063 0.063 0.125	0.063-0.25 0.133 0.125 0.25	<0.016-0.125 0.043 0.031 0.063	0.125-2 0.414 0.25 2	0.031-2 0.68 1 2	0.016-1 0.1 0.063 1
Blastomyces (n=9) GM MIC/MEC	<0.016-0.063 0.023	2->64 16	<0.016-0.125 0.029	<0.016-0.25 0.062	<0.016-0.063 0.036	0.125-0.5 0.125	0.016-1 0.18	0.016-0.063 0.017

GM: geometric mean.

For all 11 strains of the newly described genus *Emergomyces* posaconazole had the lowest GM MIC values followed by amphotericin B, itraconazole, voriconazole, isavuconazole, anidulafungin and micafungin respectively (Table 2).

Discussion

We reported the antifungal susceptibility of 24 isolates from three genera within the *Ajellomycetaceae*; *Adiaspiromyces, Blastomyces* and *Emergomyces.* All three genera analyzed in this study sporulated slowly. This feature was also reported previously for *Paracoccidioides* (23), *Emergomyces africana* (7) and *Emmonsia pasteuriana* (24). In contrast to *Blastomyces dermatitidis* and *B. gilchristii*, *Emergomyces* spp. do not constitute biosafety level 3 organism (BSL3) , and therefore work was done in a class II biosafety cabinet. *Blastomyces* was handled, according to existing recommendations, in a BSL3 facility. Antifungal susceptibility testing of thermally dimorphic fungi is often limited to the mold phase, results of which may be misleading because the yeast phase is responsible for human disease. We limited antifungal susceptibility testing to the mold phase for the following reasons (i) the pathogenic forms of these fungi differ greatly in the size (ii) conversion time for some was very long and prone to contamination, with culturing for enough material took longer than one month. Although it is a limitation that only the mold phase was tested, to our assurance recent studies of different endemic fungi using the mold and yeast phase for susceptibility testing found no differences in MICs between the two phases (8, 25).

Treatment of patients infected by members of *Emergomyces* (formerly *Emmonsia*) has been done with amphotericin B deoxycholate (26), liposomal amphotericin B (27) or combinations of amphotericin B and itraconazole (9, 10) or caspofungin (28), and voriconazole (29). In one case report, a patient infected with *Emergomyces canadensis* strain (CBS 139873=UAMH 10370) was treated with liposomal amphotericin B (12). A patient suffering from rheumatoid arthritis, infected with *Emergomyces europaeus* (*Emmonsia* sp.*)* was treated with oral itraconazole (11). A case of *Emergomyces orientalis* was treated with amphotericin B and itraconazole after shifting due to clinical failure with fluconazole (4). These successful results were in agreement with our *in vitro* findings for this particular genus.

Members of the genus *Blastomyces* had consistently low MIC/MEC values of amphotericin B, itraconazole, voriconazole, posaconazole, isavuconazole and micafungin. Our results matched with previously published data with low MIC ranges of itraconazole and high of fluconazole (16). In a comparative study of 304 strains belonging to *Blastomyces, Histoplasma* and *Coccidioides,* amphotericin B, itraconazole and voriconazole were effective *in vitro* with the MIC_{90} being the lowest for itraconazole, followed by voriconazole and amphotericin B (17). We also report low anidulafungin GM MEC and MEC ranges for *Emergomyces* and *Blastomyces.* Micafungin exhibited potent activity against the mold phases of *Emergomyces* and *Blastomyces,* but is weakly active against the yeast-forms of *H. capsulatum*, *B. dermatitidis*, and *C. immitis* (18). In contrast there was no difference between mold and yeast phase testing of *Histoplasma capsulatum* for caspofungin (25). At present, based on *in vitro* susceptibility testing it cannot be judged whether echinocandins (anidulafungin and micafungin) have clinical usefulness for dimorphic fungal infections, since for most fungi it remains uncertain which growth form correlates better with therapeutic outcome.

Our results with *Blastomyces* testing agree with recommended treatment of blastomycosis (19). Currently, there are no published treatment guidelines for patients with *Adiaspiromyces* and *Emergomyces*. Conidia of *Adiaspiromyces* do not multiply in host tissues and it is generally a self-limiting disease. In few immunocompromised patients antifungal treatment has been attempted apparently with successful outcome (30). According to published cases and international guidelines for the management of patients with dimorphic fungal infections (31, 32) it is recommended to treat with amphotericin B, followed by an azole drug (either itraconazole or fluconazole). Our *in vitro* data are in agreement with these guidelines for amphotericin B and itraconazole but not for fluconazole when testing the mold phase. In conclusion, amphotericin B appears highly active against the mold phases of all tested strains within *Ajellomycetaceae* followed by posaconazole, itraconazole, voriconazole and isavuconazole. The results of this study warrant further investigations of micafungin as a therapeutic agent for infections caused by dimorphic fungi.

Competing interests

JFM received grants from Astellas, Basilea, and Merck. He has been a consultant to Astellas, Basilea and Merck and received speaker's fees from Merck, United Medical and Gilead Sciences. All other authors declare that they have no conflict interests.

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Note: *Adiaspiromyces* was not established, the species *Adiaspiromyces crescens* is temporarily maintained as *Emmonsia crescens* and *A. terricola* is described as *Emmonsia sola* (2).

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CHAPTER 7

Discussion and summary

Discussion

Ascomycetous fungi in the order *Onygenales* have emerged as highly important causative agents of mycoses in humans and animals. Two onygenalean families, *Arthrodermataceae* and *Ajellomycetaceae,* comprise clinically important fungi. *Arthrodermataceae* is a family of dermatophytes, the causative agents of superficial infections of skin nail and hair, known as tinea or ringworm. *Ajellomycetaceae* is a family of thermally dimorphic fungi, the causative agents of subcutaneous deep systemic infections.

Based on several common features of anamorphs, gymnothecial or cleistothecial ascomata, evanescent asci, unicellular ascospores and aleuro- or arthroconidia, O*nygenales* is considered a monophyletic lineage. Using anamorph connections, ascospore ornamentation and the ability to degrade cellulose or keratin (1), Currah established four families: *Myxotrichaceae*, *Gymnoascaceae*, *Arthrodermataceae* and *Onygenaceae*, the last three being core onygenalean families. Currah's concept of *Onygenales* has been supported not only by studies on morphology of these fungi, but also by the studies on their ecology and the molecular studies. Thus, *Arthrodermataceae*, encompassing taxa with smooth ascospores, represents a well-supported lineage based on analyses of nuclear rDNA genes and chitin syntase (2-5). The phylogenetic position of *Onygenaceae*, a group of taxa with pitted ascospores, was resolved less clearly. Sequence-based phylogenies implied that this family is polyphyletic (4, 6, 7, 8), with one clade consistently recognized. This clade, comprising *Ajellomyces capsulatus* (anamorph *Histoplasma capsulatum*), *Ajellomyces crescens* (anamorph *Emmonsia crescens*), *Ajellomyces dermatitidis* (anamorph *Blastomyces dermatitidis)*, and the species of *Paracoccidioides*, was established as a separate family *Ajellomycetaceae* by Untereiner in 2004 (9).

Within these families, there were still open questions. In *Arthrodermataceae*, from the beginning of taxonomy in 1841(10) until around 1950, 350 species names were introduced, due to confused genus and species concepts. A certain level of stabilization was achieved by introduction of the three anamorphic genera, *Trichophyton*, *Epidermophyton* and *Microsporum* (11). With the dual nomenclatural system for anamorphs and teleomorphs that was a nomenclatural obligation in that period, the teleomorph genera *Arthroderma* and *Nannizzia* were introduced. Today this system has been abandoned, which required numerous name changes. Genera are now classified by phylogeny, and former teleomorph genera may now contain anamorphic species.

In chapter 2, a multilocus phylogenetic study on dermatophytes was used to answer open taxonomic questions by delineation of generic boundaries using the new set of taxonomic criteria. As already noticed in early ITS-based phylogenetic studies of Gräser, it was evident that *Arthrodermataceae* contain phylogenetically remote genera (12) and that *Trichophyton* is polyphyletic. Our revision of the taxonomy was performed with four genes in order to reach an acceptable level of nomenclatural stability.

The same approach was used for the family *Ajellomycetaceae*. In **chapter 5**, multilocus phylogeny was applied with the primary goal to establish the phylogenetic position and evolutionary relationships of newly emerging dimorphic fungi. Additionally, the two species currently maintained in *Emmonsia* were reexamined; *Emmonsia crescens* formed a clade remote from *Emmonsia parva,* which appeared to cluster in *Blastomyces*.

Although the multilocus analysis was successfully applied to resolve the main traits of dermatophyte taxonomy, the delineation of clinically important species in *Trichophyton* with the multilocus approach is still not answered. The enigma of the species complexes *T. tonsurans / T. equinum*, and *T. rubrum / T. violaceum* / *T. soudanense*, asked for combined or different methods. **In chapter 3**, these two groups of dermatophytes were studied with mass spectrometry, using LC-MS/MS methodology in an attempt to distinguish separate entities. Multilocus data

combined with study of their proteins, physiological data, sequencing of the mating loci and AFLP analysis would eventually answer these dilemmas (Kandemir Boral H and Dukik K, unpublished data).

Our multilocus analysis of dermatophytes (**chapter 2**) has revealed two new species in the newly established genus *Nannizzia*. In **chapter 4**, this polyphasic approach combining molecular, physiological and morphological methods, was used to characterize all eleven *Nannizzia* species and offer differential diagnoses for routine identification in clinical laboratories.

In *Ajellomycetaceae*, multilocus analysis of well-established and emerging dimorphic taxa resulted in new phylogeny and taxonomy of this family, with several new taxa. In **chapter 5**, new genus with five species was established, as well as a new species in *Blastomyces*. For these novel taxa which are etiological agents of systemic mycoses in mostly immunocompromised patients, *in vitro* susceptibility testing was also performed. **Chapter 6** gives MIC/MEC values for eight tested antifungals against 24 studied strains, and these values can be used as a guide to set up antifungal therapy for these pathogens.

In summary, in this thesis, the accent was placed on multilocus phylogenetic studies of both families, *Arthrodermataceae* and *Ajellomycetaceae*, resulting in new and hopefully stable phylogeny and taxonomy at the level of family, genus and species. Furthermore, alternative proteomics methods were introduced to validate species boundaries in species complexes.

Re-classification of *Arthrodermataceae*

Although the dermatophytes are among the most frequently observed organisms in biomedicine, yet there has never been stability in the taxonomy, identification and naming of the approximately 25 pathogenic species (12). Their taxonomy has been changing over the last decades, often as a result of the different methods used to identify them and to resolve their phylogenetic relationship. Based on morphology, Emmons (11) recognized three anamorphic genera, *Trichophyton*, *Epidermophyton* and *Microsporum*. With discovery of dermatophyte teleomorphs by Dawson and Gentles (13) and Stockdale (14), the genera *Arthroderma* and *Nannizzia* were introduced to accommodate them. The application of molecular methods in the last few decades revolutionized the taxonomy of dermatophytes and other fungi. The majority of the work was based on the sequencing of ribosomal DNA: small subunit region SSU (15), large subunit region LSU (3, 16), and the internal transcribed spacer region ITS (12, 17-21). Some studies were based on protein coding genes like chitin synthase I (22), beta tubulin and translation elongation factor I-A (23, 24), calmodulin (25) or their combinations (26). To prevent misclassification of dermatophytes using phenotypic characters or just a single genetic locus, a large study including nine loci has been conducted in order to choose reliable loci for phylogenetic studies. Results of this study are presented in **chapter 1**. Three standard loci, i.e. ITS, LSU and ß-tubulin, and two novel loci, *TEF3* and *RP 60S L1* taken from Stielow et al. (27) were selected for this study. The aim of the multilocus study in **chapter 2** was to recognize genera and species boundaries by comparing tree topologies. ITS and four-gene phylogenetic trees showed the same topology: clades with high bootstrap values established the genera *Trichophyton sensu stricto*, *Epidermophyton* and *Microsporum,* with four additional, pre-existing but redefined genera *Nannizzia, Lophophyton, Paraphyton,* and *Arthroderma.* The number of genera has increased, but the species relevant to routine diagnostics mainly belong to *Trichophyton*, *Epidermophyton* and *Microsporum,* which are now smaller and better defined. The genus *Trichophyton* comprises now 16 species: *T. benhamiae*, *T. bullosum*, *T. concentricum*, *T. equinum*, *T. eriotrephon*, *T. erinacei*, *T. interdigitale*, *T. mentagrophytes*, *T. quinckeanum*, *T. rubrum*, *T. schoenleinii*, *T. simii*, *T. soudanense*, *T. tonsurans*, *T. verrucosum* and *T. violaceum*. *Epidermophyton* and *Lophophyton* have one species each, *E. floccosum* and *L. gallinae* respectively. *Microsporum* and the new genus

Paraphyton have both three species: *M. audouinii*, *M. canis* and *M. ferrugineum,* and *P. cookei, P. cookiellum* and *P. mirabile* respectively. The genus *Nannizzia* has eleven species, nine listed in **chapter 2**, *N. aenigmatica*, *N. corniculata*, *N. duboisii*, *N. fulva*, *N. gypsea*, *N. incurvata*, *N. nana*, *N. persicolor* and *N. praecox*, and *N. lorica* nom.nov. and *N. polymorpha* sp. nov., listed in **chapter 4.** The genus *Arthroderma* has 21 species: *A. borellii*, *A. ciferii*, *A. cuniculi*, *A. curreyi*, *A. eboreum*, *A. flavescens*, *A. gertleri*, *A. gloriae*, *A. insingulare*, *A. lenticulare*, *A. melis*, *A. multifidum*, *A. onychocola*, *A. phaseoliforme*, *A. quadrifidum*, *A. redellii*, *A. silverae*, *A. thuringiensis*, *A. tuberculatum*, *A. uncinatum* and *A. vespertilii*. With this new taxonomy, almost all anthropophilic species and some zoophilic ones that regularly infect humans are classified in the derived clades *Trichophyton* and *Epidermophyton*. *Microsporum* is located in the middle of the tree and is probably primarily zoophilic. Prevalently geophilic species cluster in *Nannizzia* and in the genus *Arthroderma* which is the most ancestral clade. The topologies of both ITS and multilocus trees correspond to the broad ecological grouping of dermatophytes into anthropophilic, zoophilic and geophilic species, and to their clinical significance.

The latest taxonomic overview of *Arthrodermataceae* is not exhaustive in that the taxa particularly in *Trichophyton* species complexes and in the newly introduced genera will need detailed polyphasic studies to determine exact species boundaries, similar to methods applied in **chapter 4**. Additional, innovative approaches, e.g. proteomics with LC-MS/MS, or combinations of genomics, proteomics and mating studies as in the ongoing study in the *T. tonsurans / T. equinum* group, should help in understanding the clinical differences in predilection between closely related species and determine the barcoding gap. New species are expected among zoophilic and geophilic species in under-sampled habitats. Our newly established taxonomy is a good starting point for subsequent studies.

Multilocus phylogeny of novel *Ajellomycetaceae* taxa

For nearly one century, five genera of dimorphic fungi causing systemic disease in humans and other mammals were known, viz. *Coccidioides*, *Paracoccidioides*, *Blastomyces, Histoplasma* and *Emmonsia*. Due to the unusual nature of their invasive phase, and because they caused infections almost only in rodents, species of *Emmonsia* were not commonly treated with other dimorphic fungi (28). *Coccidioides,* forming endosporulating spherules in the host, was found to be phylogenetically remote from remaining dimorphic fungi: molecular studies proved that *Coccidioides* indeed belonged to the family *Onygenaceae* (29). A close relationship of *Emmonsia* to *Blastomyces* and *Histoplasma* was established by the discovery of ascomatal hyphae and hyphal coils in an Emmonsia-like fungus, which placed it in the genus of *Ajellomyces* together with *Ajellomyces dermatitidis* (*Blastomyces dermatitidis*) and *Ajellomyces capsulatus* (*Histoplasma capsulatus*) (30).

Early molecular studies of *Emmonsia* based on ITS and LSU (29) have revealed that the two species of *Emmonsia*, *E. crescens* and *E. parva*, are not closely related. *Emmonsia crescens* formed a separate, rather uniform clade, while *E. parva* was more versatile and united with *Blastomyces* on the same branch. The same study revealed existence of two undescribed species: two isolates from a lip lesion of otherwise healthy person in Israel and one isolated from a skin lesion of an HIV patient from Canada. Both cases were considered as “unusual” *Emmonsia* isolates: morphologically they resembled *Emmonsia* but the histopathology revealed a Blastomyces-like fungus in the Israeli case (31). Phylogenetically, the isolate indeed clustered within *Blastomyces*, while the case from Canada took rather isolated position (29). These cases were part of a “wave” of newly emerging cases of systemic mycoses caused by Emmonsia-like fungi (32). Common features of all these emerging fungi were that they had been isolated from humans mostly with HIV or other immunocompromised disorders, and that they had

small yeast cells as invasive form. In **chapter 5**, the case from Israel was described as type of *Blastomyces percursus,* and the case from Canada as *Emergomyces* sp. 2, later described as *Emergomyces canadensis* (33). The first laboratory-confirmed case of blastomycosis in Alberta, Canada in 1970 (34) was morphologically attributed to *Emmonsia* (29). In **chapter 5**, this isolate was reported as *Blastomyces* sp. 1, later described as *Blastomyces helicus* (33). In 1998, a case of disseminate mycosis on an HIV-infected Italian woman was reported (35). This isolate had small yeasts as invasive form and it was described as *Emmonsia pasteuriana*. In **chapter 5**, this species was described as the type of the novel genus *Emergomyces*, and was renamed as *Emergomyces pasteurianus*. Additional cases of *Emergomyces pasteurianus* were reported from Spain (36), China (37), India (38), and South Africa. The isolates from South Africa and China clustered with the type species *Emergomyces pasteurianus* in a single clade. Another *Emmonsia* sp. in a biopsy of a German patient treated with corticosteroids producing small yeasts was reported in 2003 (39). In **chapter 5** this isolate, *Emergomyces* sp. 6, later described as *Emergomyces europaeus* (33), clustered with other *Emergomyces* species. In 2013, Kenyon and co-workers (40) published 13 cases of disseminated mycosis due to another Emmonsia-like species with small yeast as invasive phase. Some of these isolates were analyzed in **chapter 5**, and described as *Emergomyces africanus*. The most recently described species of this genus, *Emergomyces orientalis*, was reported by Wang et al. (41). This species caused disseminated infection in a patient with diabetes. In the *Blastomyces* clade, along with the well-known *B. dermatitidis* / *B. gilchristii* species complex, *B. parvus* (former *Emmonsia parva*) and the newly described *B. percursus* and *B. helicus*, one more species was described, *B. silverae* (33). With these taxonomic novelties, all newly emerged species have been described, but the question of polyphyly in *Emmonsia* has not yet been solved (33). In an overall phylogeny, the genera *Blastomyces* and *Emergomyces* are separated by *Histoplasma*, while *Paracoccidioides* is ancestral to all of these dimorphic genera.

In summary, combined phylogenomics, multilocus phylogenic studies with the studies on ecology and morphology of the strains belonging to *Ajellomycetaceae* have resulted in: i) a new genus of dimorphic species, *Emergomyces* with type species *Emergomyces pasteurianus* and four newly described species, *Es. africanus***,** *Emergomyces orientalis*, *Emergomyces canadensis* and *Emergomyces europaeus*, ii) the genus *Emmonsia* with unresolved phylogenetic status (33) comprising *Emmonsia crescens* and *E. sola*, iii) the genus *Blastomyces* with *Blastomyces dermatitidis* (type species), *Blastomyces gilchristii*, *Blastomyces parvus*, *Blastomyces silverae*, *Blastomyces helicus* and *Blastomyces percursus*.

Alternative identification methods: LC-MS/MS top down mass spectrometry

The multilocus phylogeny of the members of *Arthrodermataceae* (**chapter 2**) has resulted in a classification of nearly all dermatophytes. Both ITS and four-loci phylogenetic trees showed high degree of correspondence, generating seven high-bootstrap supported clades representing the genera *Trichophyton, Epidermophyton, Nannizzia, Paraphyton, Lophophyton, Microsporum* and *Arthroderma*. With the newly proposed taxonomy, the genus *Trichophyton* comprises 16 anthropophilic and some zoophilic species. Although the molecular phylogeny approach was able to resolve the main treats of *Arthrodermataceae*, it failed in some details. This is the case with some well-established and clinically different species in *Trichophyton* which appeared to be genetically indistinguishable in the multilocus analysis presented in **chapter 2**. Chen et al (42) introduced the term 'species complex' for populations that are doubtfully distinct. In *T. mentagrophytes* series (**chapter 2**), anthropophilic and zoophilic species form undistinguishable species complexes, as is the case of *Trichophyton tonsurans / T. equinum*, and *T. rubrum / T. violaceum / T. soudanense*. These are important species in medical mycology and

veterinary medicine, and thus alternative methods should be developed in order to determine species boundaries. Since these species could as yet not be distinguished genetically, study of their proteins might explain different host and clinical predilections. **In chapter 3**, one such proteomic method was tested in an attempt to differentiate *Trichophyton tonsurans / T. equinum* (human / horse predilection) and *T. rubrum / T. violaceum* / *T soudanense* (tinea corporis + tinea pedis *versus* tinea capitis predilection). *Trichophyton interdigitale* was included in the analysis as closest but still distinguishable species to the *T. tonsurans / T equinum* complex. The method used was innovative top-down liquid chromatography tandem mass spectrometry, LC-MS/MS. By choosing the two *Trichophyton* complexes, the goal of the study was to test its resolution power, and compare it to ITS sequencing (as a gold standard) and MALDI-TOF. The results of this study were encouraging: i) three species were identified down to the species level, *T. rubrum* / *T. violaceum* (including *T. soudanense*) *versus T. interdigitale*, ii) all strains of the identified three species and 4 out of 12 strains of *T. tonsurans / T equinum* complex were identified to strain level, emphasizing the potential of this method in microbial identification in clinical epidemiology, iii) 6, 2, 3, 3, 5, 2 unique masses were discovered for: *T. rubrum* / *T violaceum, T. tonsurans / T. equinum / T. interdigitale, T. tonsurans / T. equinum, T. equinum / T. interdigitale, T. interdigitale* and *T. tonsurans,* respectively (**chapter 3**).

Trichophyton interdigitale is routinely distinguished from *T. tonsurans* and *T. equinum* with ITS sequencing (9 nucleotides difference) and the two strains in our study were also easily distinguished at species and strain level, having 5 unique masses which were not present in any other species. For *T. rubrum / T. violaceum* complex, LC-MS/MS is comparable to ITS sequencing in species identification, and it performs better than MALDI-TOF, where misidentifications were reported in several studies (43-46). For *T. tonsurans / T. equinum* complex, species identification with LC-MS-MS showed that 8 out of 12 strains had highly overlapping proteomes regardless of the host, confirming the unresolved taxonomy which was already demonstrated with ITS sequencing and the phylogenetic data presented in **chapter 2**. This complex was studied with MALDI-TOF by Nenoff et al. (47) and De Respins et al. (48). These authors claimed successful species identification, but did not specify on which ground they denominated the strains as *T. tonsurans* or *T. equinum*. Our data of overlapping proteomes and the inconsistency in T/C SNP in ITS not corresponding with host range show that their separation is highly controversial. To solve this question, additional studies have been started on a larger dataset and adding AFLP, MAT locus sequencing, physiological studies and mating experiments to the proteomics data and ITS sequences (Kandemir Boral H, Dukik K, unpublished data).

Nannizzia species: Polyphasic approach

With the newly proposed taxonomy of *Arthrodermataceae* **(chapter 2)**, nine species were included in the genus *Nannizzia: N. aenigmatica*, *N. corniculata*, *N. duboisii*, *N. fulva*, *N. gypsea*, *N. incurvata*, *N. nana*, *N. persicolor*, and *N. praecox*. The study revealed two additional members of this genus, *N. lorica* nom. nov. and *N. polymorpha* sp. nov. The aim of the study in **chapter 4** was to fully characterize these eleven *Nannizzia* species using: i) ITS, LSU, *TUB2*, *TEF3* and *RP60S L1*-based phylogeny, ii) calculation of sequence similarities for these loci, iii) morphological and physiological features. The results would offer a comprehensive differential diagnosis that can be used for identification of these species in clinical laboratories with basic and advanced identification facilities.

Phylogenetic analyses of the family *Arthrodermataceae* based on ITS and the concatenated dataset of *Nannizzia* strains were highly congruent, showing similar phylogenetic relationships among *Nannizzia* species. The phylogenetic tree of *Nannizzia* (**chapter 4**) has high bootstrap values: branches between clades have BS above 70% and clades representing seven species

have BS of 100%.

Identity matrices of five loci (**chapter 4**) showed that in all loci except LSU the distances between the species are rather large; *TUB2* and *RP 60S L1* loci are the most discriminative, followed by ITS and *TEF3*. The summarized morphological and physiological features are insufficiently informative for species delimitation but in combination they can still be used for routine identification of main species in laboratories with limited molecular facilities. As in many other fungal groups, sequencing of diagnostic loci is superior to classical phenotypic identification.

In vitro antifungal susceptibility testing

In **chapter 6,** the results of the first *in vitro* susceptibility testing of newly described species *Emergomyces africanus, Es. orientalis, Es. canadensis*, *Blastomyces percursus, B. helicus* and *B. silverae* were presented. In total, the filamentous forms of 24 strains representing 11 species of 3 dimorphic genera, *Emmonsia*, *Emergomyces* and *Blastomyces,* were tested using eight antifungals, viz. amphotericin B (AMB), fluconazole (FLC), itraconazole (ITC), voriconazole (VOR), posaconazole (POS), isavuconazole, (ISA), anidulafungin (ANID), and micafungin (MICA), following guidelines of the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document.

The results for the known species, *Es. pasteurianus*, *B. dermatitidis* and *B. gilchristii* were correlated to previously published data (38, 49). MIC ranges for the eight antifungal drugs for all three genera were similar; high MIC for FLC, and low for the other azoles. Amphotericin B was the most active drug followed by posaconazole, itraconazole, voriconazole and isavuconazole. MIC values for ISA were in range with the other azoles but higher for *Emergomyces* than for the other genera. The echinocandins MEC values were higher than those of azoles and AMB. Micafungin demonstrated good *in vitro* antifungal activity against *Emergomyces* and *Blastomyces* strains**.**

This is the first comparative study of *in vitro* susceptibility for the novel and known species in *Emmonsia, Emergomyces* and *Blastomyces*. MIC values for all 8 antifungal drugs are comparable, and in agreement with treatment guidelines for dimorphic fungi (50, 51). The obtained results might help the clinicians to decide upon optimal treatment of infections caused by these fungi, and establish standardization of *in vitro* susceptibility testing for dimorphic fungi.

General conclusions

This thesis focuses on the taxonomy of dermatophytes and emerging dimorphic fungi, searches for the best (secondary) locus for identification and phylogeny of these important onygenalean families, and tests LC-MS/MS as an alternative method for identification. For both *Arthrodermataceae* and *Ajellomycetaceae,* a thorough phylogenetic study based on four and five loci, respectively, was performed, which resulted in demarcation of some species boundaries, re-classification of others, and introduced new genera and species. Locus assessment has revealed that ITS is among the best markers for identification and phylogenetic study for *Arthrodermataceae*, and *rPB2* for *Ajellomycetaceae* while *TUB2* is the second best for both families. For species complexes and pairs or groups of species with high levels of genetic similarity, LC-MS/MS was used to supplement or exchange molecular characterization. For full characterization of the (novel) taxa, all identification methods should be combined. First results of antifungal susceptibility testing of the newly described dimorphic fungi are important to guide and establish clinical treatment protocols for dimorphic fungi, especially in cases where reference methods for these important systemic pathogens are lacking.

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Appendix

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Curriculum vitae

Karolina Dukik was born in Kochani, Republic of Macedonia on 13th August 1969. She finished the primary and secondary school (major biotechnology) in her home city. She continued her education is Skopje, at the University of Sts "Cyril and Methodius" the faculty of Agriculture. After completing the bachelor studies there were several years of struggle to keep up with the difficult situation in her country, due to the war and a bad situation in the whole region of former Yugoslavia. But the love for science never stopped, and in 1999 she started the postgraduate studies in molecular biology at the Macedonian Academy of Sciences and Arts (MANU). These studies were EU support for talented students with the goal to introduce modern molecular biology techniques to the participating faculties and give the oportunities to learn more from the visiting professors. Here is where she found out what is going to be her future profession, working in science. During these studies she met a Dutch professor from Wageningen University and got a grant to do a six months internship at the deparment of Nematology at WUR. Coming to the Netherlands in 2000 was a major turning point in her life: two loves combined, the one for science and the one for the country and the Dutch people. With all exams taken and a written master thesis Karolina was not able to finish her postgraduate studies in Macedonia due to administrative problems. She decided to come back to Wageningen and start again master studies in Plant Biotechnology, major Phytopathology. In 2005 she got her Master degree with two major theses, one in Nematology and one in Phytopathology. She continued almost immediately her work in science as a research assistant in Add2X Biosciences B.V, a spin off company at Leiden University for three years. In 2008 she decided to find a job in Utrecht were she moved a year earlier. In 2008 she applied for a job at CBS and started working as a techician in the collection for four years. During this years her wish to go further in science came back again and due to the personal support from Sybren de Hoog and his collaboration with Thermo Fisher Scientific she got a position of a PhD candidate in his research group. Five years of learning process and hard work and this is a result of her journey; this thesis is a crown of her academic career and the biggest scientific gift of her life.

List of publications

Dukik K, Munoz JF, Jiang Y, Feng P, Sigler L, Stielow JB, Freeke J, Jamalian A, Gerrits van den Ende B, McEwen JG, Clay OK, Schwartz IS, Govender NP, Maphanga TG, Cuomo CA, Moreno LF, Kenyon C, Borman AM, de Hoog S. 2017. Novel taxa of thermally dimorphic systemic pathogens in the Ajellomycetaceae (Onygenales). Mycoses 60:296-309.

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Contributions to ongoing projects

Kandemir H, Dukik K, Hagen F, de Hoog GS. Species boundaries in T. tonsurans complex (publication in preparation)
Delma-Zohra F, Dukik K, Kandemir H, Stielow B, de Hoog GS. Molecular phylogeny of the order Onygenales (phylogenetic analysis completed)
Danesi P, Dukik K, de Hoog GS. Detection of Onygenales in wild animals (publication in preparation)
Rezaei-Matehkolaei A, Ajafzadeh J, Dukik K, de Hoog GS. New Iranian dermatophytes species close to Arthroderma benhamiae (phylogenetic analysis completed)
Rezaei-Matehkolaei A, Najafzadeh MJ Dukik K, de Hoog S. Iranian Aphanoascus species (phylogenetic analysis completed)
Araujo M, Dukik K, de Hoog S. Onygenalean fungal species in Brazilian beach soil (phylogenetic analysis completed)

Nederlandse samenvatting

Dit proefschrift behandelt twee families pathogene schimmels in de orde Onygenales, de *Arthrodermataceae* en de *Ajellomycetaceae*. Monofylie van deze families binnen Onygenales is al meermaals bevestigd, maar de relatie tussen de genera en de afbakening van soorten is nog niet opgelost. De laatste decennia zijn veel moleculaire studies gewijd aan de resolutie potentie van verschillende markers. Algemene conclusie was dat taxonomie op grond van één enkele locus uit de begintijd van de moleculaire toepassing niet goed mogelijk is, in het bijzonder voor afbakening van taxa en voor evolutionaire en taxonomische conclusies.

Om classificatie van dermatofyten op grond van fenotypische kenmerken of met slechts een genetische locus te verbeteren is onderzoek gedaan met behulp van negen loci, om daar vervolgens betrouwbare markers voor fylogenetische studies te selecteren. De resultaten van deze studie worden gepresenteerd in **hoofdstuk 1**. Drie standaard-loci, d.w.z. ITS, LSU en ß-tubuline, en twee nieuwe loci, TEF3 en RP 60S L1 werden gebruikt voor nadere analyse. In dit multilocus onderzoek van dermatofyten in **hoofdstuk 2** werden genus- en soortsgrenzen bepaald via een vergelijking van topologieën van de fylogenetische bomen. Fylogenetische bomen gebaseerd op ITS en op 4 partiële coderende genen vertoonden dezelfde topologie: clades met hoge bootstrap-waarden bevestigden de genera *Trichophyton sensu stricto, Epidermophyton* en *Microsporum*, plus de vier reeds bestaande maar opnieuw gedefinieerde genera *Nannizzia, Lophophyton, Paraphyton* en *Arthroderma*. Het aantal genera is groter dan vroeger, maar de relevante soorten in routine diagnostiek behoren meestal tot de bekende genera *Trichophyton, Epidermophyton* en *Microsporum*, die nu kleiner en overzichtelijker zijn.

Het genus *Trichophyton* bevat nu 16 soorten: *T. benhamiae*, *T. bullosum*, *T. concentricum*, *T. equinum*, *T. eriotrephon*, *T. erinacei*, *T. interdigitale*, *T. mentagrophytes*, *T. quinckeanum*, *T. rubrum*, *T. schoenleinii*, *T. simii*, *T. soudanense*, *T. tonsurans*, *T. verrucosum* en *T. violaceum*. *Epidermophyton* en *Lophophyton* bevatten elk één soort, *E. floccosum* en *L. gallinae,* respectievelijk. *Microsporum* en het nieuwe genus *Paraphyton* hebben drie species elk: *M. audouinii*, *M. canis* and *M. ferrugineum,* resp. *P. cookei, P. cookiellum* en *P. mirabile*. Het genus *Nannizzia* bevat 11 species, waarvan 9 zijn opgenomen in **hoofdstuk 2**: *N. aenigmatica*, *N. corniculata*, *N. duboisii*, *N. fulva*, *N. gypsea*, *N. incurvata*, *N. nana*, *N. persicolor* en *N. praecox*. *Nazzizzia lorica* nom. nov. en *N. polymorpha* sp. nov. zijn nieuw, opgenomen in **hoofdstuk 4.** Het genus *Arthroderma* heeft 21 species: *A. borellii*, *A. ciferii*, *A. cuniculi*, *A. curreyi*, *A. eboreum*, *A. flavescens*, *A. gertleri*, *A. gloriae*, *A. insingulare*, *A. lenticulare*, *A. melis*, *A. multifidum*, *A. onychocola*, *A. phaseoliforme*, *A. quadrifidum*, *A. redellii*, *A. silverae*, *A. thuringiensis*, *A. tuberculatum*, *A. uncinatum* and *A. vespertilii*. Met deze nieuwe taxonomie zijn bijna alle anthropofiele soorten, en sommige zoofiele soorten met een associatie met de mens geclassificeerd in de evolutionair recente *Trichophyton* en *Epidermophyton* clades. *Microsporum* bevindt zich in het midden van de boom en is waarschijnlijk in de eerste plaats zoofiel. Prevalent geofiele soorten bevinden zich in *Nannizzia* en in het genus *Arthroderma,* de meest oorspronkelijke clade.

Hoewel de multilocus analyse met succes is toegepast om de belangrijkste kenmerken van dermatofyten te herdefiniëren, is de afbakening van klinisch belangrijke soorten in *Trichophyton* ondanks multilocus analysen nog steeds onduidelijk. Het enigma van de soortencomplexen *T. tonsurans / T. equinum*, en *T. rubrum / T. violaceum / T. soudanense* maakt combinatie van verschillende methoden noodzakelijk. In **hoofdstuk 3** werden deze twee groepen dermatofyten bestudeerd met LC tandem-massaspectrometrie, in een poging om afzonderlijke entiteiten te onderscheiden. In deze studie konden *T. rubrum, T. violaceum* en *T. interdigitale* correct tot op soortniveau worden geïdentificeerd – of zelfs tot op stam-niveau. In het *T. tonsurans / T. equinum*-complex hadden 8 van de 12 stammen bijna identieke proteomen, en derhalve kon het verschil tussen deze twee soorten niet worden opgelost.

Zoals hierboven vermeld heeft de multilocus-analyse van dermatofyten (**hoofdstuk 2**) twee

nieuwe soorten opgeleverd in het opnieuw gedefinieerde geslacht *Nannizzia*. In **hoofdstuk 4** werd een polyfasische benadering gebruikt waarbij moleculaire, fysiologische en morfologische methoden werden gecombineerd om alle elf *Nannizzia*-soorten te karakteriseren en differentiële diagnoses te bieden voor identificatie in klinische routine laboratoria. Vergelijking van vijf loci (**hoofdstuk 4**) toonde aan dat met alle loci, behalve LSU, de afstanden tussen de soorten aanzienlijk zijn. TUB2 en RP 60S L1 hebben het meest discriminerend vermogen, gevolgd door ITS en TEF3. Morfologische en fysiologische kenmerken zijn onvoldoende informatief voor afbakening van species, maar in combinatie kunnen ze nog steeds worden gebruikt voor routine diagnostiek van meest voorkomende soorten in laboratoria met beperkte moleculaire voorzieningen. Zoals in veel andere schimmelgroepen, is de sequentiëring van diagnostische loci aanzienlijk beter dan klassieke fenotypische identificatie.

In de familie *Ajellomycetaceae* resulteerde multilocus-analyse van klassieke en emergente dimorfe taxa in een nieuwe fylogenie en taxonomie, met verschillende nieuwe species. In **hoofdstuk 5** heeft combinatie van phylogenomics en multilocus analyse met ecologische en morfologie data geresulteerd in: i) een nieuw geslacht van dimorfe fungi, *Emergomyces*, gebaseerd op type soort *Emergomyces pasteurianus* met daarnaast vier nieuwe soorten, *E. africanus, E. orientalis, E. canadensis* en *E. europaeus*; ii) het geslacht *Emmonsia* met *Emmonsia crescens* en *Emmonsia sola* kon nog niet bevredigend worden geclassificeerd, iii) nieuwe definitie van het geslacht *Blastomyces*, met *Blastomyces dermatitidis* (type soort), *Blastomyces gilchristii, Blastomyces parvus, Blastomyces silverae, Blastomyces helicus* en *Blastomyces percursus*.

In **hoofdstuk 6** wordt de *in vitro* antifungale gevoeligheid van nieuw beschreven soorten bepaald, nl. *Emergomyces africanus, E. orientalis, E. canadensis*, *Blastomyces percursus, B. helicus* en *B. silverae*. In totaal werden de filamenteuze vormen van 24 stammen van 11 soorten getest in de drie dimorfe geslachten *Blastomyces*, *Emergomyces* en *Emmonsia*. Acht antischimmelmiddelen werden gebruikt, nl. amfotericine B (AMB), fluconazol (FLC), itraconazol (ITC), voriconazol (VOR), posaconazol (POS), isavuconazol, (ISA), anidulafungin (ANID) en micafungine (MICA), volgens de richtlijnen van het M38-A2-document van het Clinical and Laboratory Standards Institute (CLSI). MIC-waarden voor alle acht middelen waren vergelijkbaar: hoge MIC voor FLC en lage waarden voor de overige azolen. Amfotericine B was de meest actieve stof gevolgd door posaconazol, itraconazol, voriconazol en isavuconazol. MIC-waarden voor ISA lagen binnen de variatiebreedte van de andere azolen, maar waren hoger voor *Emergomyces* dan voor de andere geslachten. De MIC-waarden van echinocandinen waren hoger dan die van azolen en AMB. Micafungine vertoonde goede *in vitro* activiteit tegen *Emergomyces*- en *Blastomyces*-stammen.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)
- Thermofisher research group

Post-graduate courses (3.6 ECTS)
- Emerging Zygomyces; ECMM/ISHAM-zygomycetes (2013)
- Current trends in phylogenetics; EPS-WUR (2013)
- Training-photophop for publications; CBS (2018)
- Training INDesign for thesis; CBS (2018)

Laboratory training and working visits (3 ECTS)
- Medical mycology training; Sun-yat-Sen University China in collaboration with CBS (201

Invited review of (unpublished) journal manuscript (2 ECTS)
- Medical Mycology: taxonomy of (2017)
- Scientific Reports: genome analysis reveals evolutionary mechanisms of adaptation in syst dimorphic fungi (2017)

Deficiency, refresh, brush-up courses (3 ECTS)
- Biomolecular mass spectrometry course; UU (2015)

Competence strengthening / skills courses (4.3 ECTS)
- Workshop personal leadership; Chiat Cheong (2016)
- Exploring teaching outside academia; WGS (2018)

PE&RC Annual meetings, seminars and the PE&RC weekend (0.9 ECTS)
- Last year PE&RC weekend (2017)
- Annual PE&RC day (2017)

Discussion groups / local seminars / other scientific meetings (8.7 ECTS)
- Barcoding Masterclass (2013)
- Monday symposia at Westerdijk Institute (2013-2016)
- Diversity and Barcoding of Medical Fungi (2014)
- ISHAM workshop MALDI-TOF (2015)
- Genomics of neglected pathogens (2015)
- ISHAM Workshop on dermatophytes (2016)

International symposia, workshops and conferences (13.2 ECTS)
- 19th ISHAM Congress; oral presentation (2015)
- 26th ECCMID Congress; poster presentation (2016)
- 27th ECCMID Congress; poster presentation (2017)

- 8th Trends in Medical Mycology; oral presentation (2017)
- 20th ISHAM Congress; 2 oral presentations (2018)

Lecturing / Supervision of practicals / tutorials (1.5 ECTS)

- Towards a multilocus phylogeny of dermatophytes (2014)
- Redefining the genus *Emmonsia*; emergence of new dimorphic human pathogens (2015)
- Molecular characterization of *Trichophyton* species using multi locus based phylogeny and novel proteomics approaches (2016)
- Elements of reclassification of dermatophytes (2016)
- Reclassification of *Arthrodermataceae* (2016)

Supervision of MSc students (3 ECTS)

- Phylogeny of *T. rubrum* complex
- Solving on *T. tonsurans* complex puzzle
- New taxa in *Ajellomycetaceae*