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## Resolving the taxonomy of emerging zoonotic pathogens in the *Trichophyton benhamiae* complex

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**Resolving the taxonomy of emerging zoonotic pathogens in the *Trichophyton benhamiae* complex**

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16

17 **Running head:** Emerging pathogens in the *Trichophyton benhamiae* complex

18

## 19 **Abstract**

20 Species of the *Trichophyton benhamiae* complex are predominantly zoophilic pathogens with a  
21 worldwide distribution. These pathogens have recently become important due to their epidemic spread  
22 in pets and pet owners. Considerable genetic and phenotypic variability has been revealed in these  
23 emerging pathogens, but the species limits and host spectra have not been clearly elucidated. In this  
24 study, we used an approach combining phylogenetic analysis based on four loci, population-genetic  
25 data, phenotypic and physiological analysis, mating type gene characterization and ecological data to  
26 resolve the taxonomy of these pathogens. This approach supported the inclusion of nine species in the  
27 complex, including three new species and one new variety. *Trichophyton benhamiae* var. *luteum* var.  
28 nov. (“yellow phenotype” strains) is currently a major cause of zoonotic tinea corporis and capitis in  
29 Europe (mostly transmitted from guinea pigs). This variety exhibits unique phenotypic and ecological  
30 characteristics compared to *T. benhamiae* var. *benhamiae* and is distinguishable by using microsatellite  
31 markers but not with the conventional DNA sequence markers used here. We demonstrated that isolates  
32 of the “white phenotype” do not form a monophyletic group and are segregated into *T. benhamiae* var.  
33 *benhamiae* (mostly from North America; dogs), *T. europaeum* sp. nov. (mostly from Europe; guinea  
34 pigs), and *T. japonicum* sp. nov. (the major cause of zoonotic infections in Japan but also found in  
35 Europe; rabbits and guinea pigs). The name *T. africanum* sp. nov. is proposed for the “African” race of  
36 *T. benhamiae*. The extinction of one mating type gene and adaptation to different hosts have played  
37 important roles in the evolution of pathogens from the *T. benhamiae* complex. A microsatellite typing

1 scheme consisting of ten markers was developed for the purpose of the epidemiological surveillance of  
2 these emerging pathogens. MALDI-TOF MS was able to discriminate between the newly proposed  
3 species and varieties, suggesting that this method is useful for identification in clinical practice.  
4

## 5 **INTRODUCTION**

6 Dermatophytes are a group of fungal pathogens that cause inflammatory and contagious skin diseases  
7 that are usually referred to as dermatophytoses, tinea or ringworm. These are among the most common  
8 diseases of warm-blooded animals, including humans, and their prevalence can reach dozens of percent  
9 in both human and animal populations (Havlickova et al. 2008, Seebacher et al. 2008, Cafarchia et al.  
10 2010, Duarte et al. 2010, Agnetti et al. 2014, Ahdy et al. 2016, Kupsch et al. 2017). The treatment and  
11 prevention of these infections in humans, companion animals and pets require a considerable amount  
12 of funding every year (Kane and Summerbell 1997, Chermette et al. 2008, Bond 2010, Benedict et al.  
13 2018, Shenoy and Jayaraman 2019).

14 The incidence of zoonotic dermatomycoses transmitted to humans from livestock decreased  
15 significantly in developed countries with the intensification of agriculture, introduction of preventive  
16 measures (e.g., vaccination in cattle) and advances in treatment options (Borman et al. 2007, Lund et  
17 al. 2014). In contrast, zoonotic infections transmitted from pets remain an important public health  
18 concern worldwide (Hubka et al. 2018c). *Microsporum canis* and *Trichophyton mentagrophytes* remain  
19 major agents of dermatophytosis in many domestic animals and cause a significant number of zoonotic  
20 dermatophytoses in humans (Hayette and Sacheli 2015). In addition to these well-known causal agents,  
21 several emerging zoonotic pathogens are increasingly reported in both humans and pets, and most of  
22 them belong to the *Trichophyton benhamiae* complex.

23 The *Trichophyton benhamiae* complex currently comprises six species: *T. benhamiae*, *T.*  
24 *bullosum*, *T. concentricum*, *T. erinacei*, *T. eriotrephon* and *T. verrucosum* (Lysková et al. 2015, de  
25 Hoog et al. 2017). These species are predominantly zoophilic, with the exception of anthropophilic *T.*  
26 *concentricum*, an agent of tinea imbricata in tropical regions (Bonifaz et al. 2004, Pihet et al. 2008,  
27 Bonifaz and Vazquez-Gonzalez 2011). *Trichophyton verrucosum*, a cause of dermatophytosis in cattle  
28 and other ruminants, is one of the best-known members of the complex. It has a worldwide distribution  
29 and causes economic losses in the food (negative impacts on milk and meat production), hide and skin  
30 industries (Chermette et al. 2008, Bond 2010). The incidence of infections in cattle has decreased in  
31 many regions in response to vaccination programmes or changes in agricultural systems, and the rate  
32 of infections in humans has decreased proportionally (Seebacher et al. 2008; Lund et al. 2014). By  
33 contrast, a lack of prophylaxis accounts for the high infection rates observed in countries such as Italy  
34 (Moretti et al. 2013). *Trichophyton verrucosum* grows slowly in culture and frequently produces only  
35 chlamydospores as its main microscopic characteristic. In this respect, it is superficially very similar to  
36 *T. bullosum*, which causes infections in donkeys and horses, but is much less common and is  
37 geographically restricted to the Middle East, Africa and Europe (Sitterle et al. 2012, Lysková et al.

1 2015, Sabou et al. 2018). Scant data are available on the distribution of *T. eriotrephon*, which is only  
2 known from several poorly documented cases of dermatophytosis in humans and dogs (Rezaei-  
3 Matehkolaei et al. 2013, Hubka et al. 2018c, Sabou et al. 2018). The remaining two zoophilic species,  
4 *T. benhamiae* and *T. erinacei*, are currently considered emerging pathogens, as their incidence as a  
5 cause of infections in pets and humans has increased significantly in the last decade (Hubka et al.  
6 2018c).

7         A strikingly high incidence of zoonotic *T. benhamiae* (syn. *Arthroderma benhamiae*) infections,  
8 contracted mostly from guinea pigs, is currently reported in various European countries. Although this  
9 species was considered less clinically important in recent decades, it became one of the most common  
10 agents of zoonotic dermatophytoses after 2010 (Symoens et al. 2013, Nenoff et al. 2014, Uhrlaß et al.  
11 2015, Hubka et al. 2018b, Sabou et al. 2018). It has been shown that the prevalence of the pathogen in  
12 guinea pig breeds and pet shops reaches up to 90 % (Drouot et al. 2009, Kupsch et al. 2017, Overgaauw  
13 et al. 2017, Guillot et al. 2018, Bartosch et al. 2019). Infections occur more frequently in young guinea  
14 pigs and are usually asymptomatic. The presence of skin lesions with hair loss (mostly on the muzzle,  
15 forehead, ears and around eyes) is also reported in some individuals (Kraemer et al. 2012, Kraemer et  
16 al. 2013). When transmitted to the human host, the infections manifest most commonly as highly  
17 inflammatory tinea of glabrous skin and tinea capitis and less commonly as onychomycosis (Nenoff et  
18 al. 2014, Skořepová et al. 2014). The presence of asymptomatic infections in animal hosts contributes  
19 to the successful spread of the pathogen between animals kept in groups. Such asymptomatic infections  
20 also facilitate transmission to pet owners and the occurrence of small familial outbreaks or general  
21 infections among pet breeders, pet shop workers and others. In addition to guinea pigs, this pathogen  
22 has been reported in dogs, rabbits, cats, North American porcupines, various small rodents and foxes  
23 (Aho 1980, Fréalle et al. 2007, Takeda et al. 2012, Sieklucki et al. 2014, Hiruma et al. 2015, Ziółkowska  
24 et al. 2015, Needle et al. 2019).

25         *Trichophyton benhamiae* was originally described from several dog and human infections in  
26 North America (Ajello and Cheng 1967). The same authors induced a sexual state of the fungus and  
27 demonstrated its heterothallic nature by using *in vitro* mating experiments. In subsequent years,  
28 Takashio (1974) recognized two races among strains of *T. benhamiae* based on biological compatibility  
29 experiments: an “Americano-European” race and an “African” race of *Arthroderma benhamiae*  
30 (Takashio 1974). Furthermore, two phenotypically different groups among strains of the Americano-  
31 European race have recently been recognized by different authors and designated the “yellow  
32 phenotype” and “white phenotype” strains (Symoens et al. 2013, Nenoff et al. 2014, Hiruma et al. 2015,  
33 Brasch et al. 2016). The characterization of mating type genes showed that the MAT1-1-1 idiomorph  
34 was significantly prevalent among strains of the yellow phenotype, while MAT1-2-1 prevailed among  
35 strains of the white phenotype (Symoens et al. 2013). Similar observations of a lack of one MAT gene  
36 or significant bias towards one MAT idiomorph have been made in several other primary pathogenic

1 dermatophytes, while the prevalence of both mating types in a balanced ratio is common in geophilic  
2 species (Metin and Heitman 2017, Kosanke et al. 2018).

3 It was demonstrated that the vast majority of European infections are caused by yellow  
4 phenotype strains that emerged relatively recently (Symoens et al. 2013, Hubka et al. 2014, Nenoff et  
5 al. 2014, Uhrlaß et al. 2015). The first documented cases of infections due to yellow phenotype strains  
6 were recorded between 2002 and 2008 in France and Switzerland (Contet-Audonneau and Leyer 2010,  
7 Charlent 2011, Khettar and Contet-Audonneau 2012, Symoens et al. 2013). The first cases in Germany  
8 and the Czech Republic were described shortly before 2010, and the pathogen became rapidly epidemic  
9 during the following years. Currently, *T. benhamiae* is the most important agent of dermatophytoses  
10 transmitted from animals in the Czech Republic and Germany (Hubka et al. 2014, Nenoff et al. 2014,  
11 Uhrlaß et al. 2015, Hubka et al. 2018b, Kupsch et al. 2020). The origin of yellow phenotype strains of  
12 *T. benhamiae* and the reason for the sudden increase in the incidence of human and animal infections  
13 in Europe after 2010 are unknown. As the breeding of guinea pigs has been popular in Europe for  
14 decades, the epidemic cannot be explained by a change in pet owner behaviour. Therefore, the spread  
15 of a new virulent and highly transmissible genotype/lineage was hypothesized (Čmoková 2015, Hubka  
16 et al. 2018c). The occurrence of *T. benhamiae* infections in non-European countries is generally poorly  
17 known except for individual reported cases. This is mostly due to insufficient surveillance and a lack  
18 of long-term epidemiological studies supported by molecular-based identification of dermatophytes.

19 In contrast to yellow phenotype strains, white phenotype strains have probably existed long  
20 term worldwide. Sporadic human and animal infections due to white phenotype strains were described  
21 from various European countries, Japan and the USA before the widespread dispersal of yellow  
22 phenotype strains in Europe (Ajello and Cheng 1967, Takashio 1974, Aho 1980, Hejtmánek and  
23 Hejtmánková 1989, Kano et al. 1998). In Japan, white phenotype strains were first reported in 1996  
24 from an infected rabbit (Kano et al. 1998); human cases were reported in the following years (Nakamura  
25 et al. 2002), and the infections were summarized by Kimura et al. (2015). The increasing number of  
26 people breeding pets, together with the increasing import of animals to Japan, is considered a cause of  
27 the increased incidence in Japan (Hiruma et al 2015, Kimura et al 2015, Takeda et al 2012). Chronology  
28 of reports of white and yellow phenotype strains in various countries is summarized in Figure 1.

29 The aim of this study was to elucidate the species boundaries, host spectrum, and population  
30 structure of emerging pathogens in the *Trichophyton benhamiae* complex. We examined a large set of  
31 clinical isolates associated with human and animal infections that were mostly collected in European  
32 countries but also in the USA and Japan. We conducted DNA sequencing of four genetic loci,  
33 phylogenetic analyses, and analyses of morphology and physiology to examine whether the previously  
34 detected level of phenotypic and genetic variability reflects undescribed species diversity or a high level  
35 of infraspecific variability. The levels of recombination/clonality within species and populations,  
36 respectively, were estimated by calculating the index of association and determining the ratios between  
37 MAT locus idiomorphs. MALDI-TOF MS spectra were compared between species of the *T. benhamiae*

1 complex to test the possibility of their differentiation in the clinical setting. A set of highly variable  
2 microsatellite markers were developed to analyse the population structure and relationships between  
3 strains with differences in their geographic origin, host spectrum and phenotype. The new taxonomic  
4 classification and microsatellite typing scheme proposed in this study will enable the monitoring of  
5 changes in the frequencies of individual species and genotypes. It will help to evaluate the results of  
6 preventive measures and interventions and is a basic prerequisite for the development of  
7 epidemiological studies.

## 9 **MATERIALS AND METHODS**

### 10 **Source of isolates**

11 More than three hundred strains isolated from human and animal patients with dermatophytosis caused  
12 by *T. benhamiae* complex species were obtained for this study from various clinical laboratories,  
13 hospitals and universities (Table S1): Laboratory for Medical Microbiology (Mölbis, Germany),  
14 College of Veterinary Medicine, University of Illinois at Urbana-Champaign (USA), The University of  
15 Tokyo (Japan), School of Veterinary Medicine, University of Turin (Italy), and various institutions in  
16 the Czech Republic (Institute of Public Health in Ostrava and Prague, General University Hospital in  
17 Prague, University Hospital in Pilsen, Hospital České Budějovice, Hospital in Pardubice and Labvet  
18 veterinary laboratory in Prague). This set of strains was further supplemented with isolates from culture  
19 collections, especially BCCM/IHEM Biomedical Fungi and Yeasts Collection (Brussels, Belgium) and  
20 CBS culture collection housed at the Westerdijk Institute (Utrecht, The Netherlands).

21 Selected isolates were deposited into the Culture Collection of Fungi (CCF), Department of  
22 Botany, Charles University, Prague, Czech Republic; herbarium specimens of newly described species  
23 were deposited into the herbarium of the Mycological Department, National Museum in Prague, Czech  
24 Republic (PRM).

### 26 **Molecular studies**

27 DNA was extracted from seven-day-old colonies using the ArchivePure DNA Yeast and Gram2+  
28 Isolation Kit (5 PRIME Inc., Gaithersburg, Maryland) according to the manufacturer's instructions as  
29 updated by Hubka et al. (2015b). The quality of the extracted DNA was evaluated by NanoDrop 1000  
30 Spectrophotometer.

31 The ITS rDNA region (ITS1-5.8S-ITS2 cluster) was amplified using the primer set SR6R and  
32 LR1 (White et al. 1990) or ITS1F and ITS4 (White et al. 1990, Gardes and Bruns 1993), partial *gapdh*  
33 gene encoding glyceraldehyde-3-phosphate dehydrogenase was amplified with primers GPDF and  
34 GPDR (Kawasaki et al. 2011), partial *tubb* gene encoding  $\beta$ -tubulin with primers Bt2a and Bt2b (Glass  
35 and Donaldson 1995), and *tefla* gene encoding translation elongation factor 1- $\alpha$  with primers EF-  
36 DermF and EF-DermR (Mirhendi et al. 2015). All primer combinations are listed in Table S2. Reaction  
37 volume of 20  $\mu$ L contained 1  $\mu$ L (50 ng mL<sup>-1</sup>) of DNA, 0.3  $\mu$ L of both primers (25 pM mL<sup>-1</sup>), 0.2  $\mu$ L

1 of My Taq Polymerase and 4 µL of 5× My Taq PCR buffer (Bioline, London, UK). PCR conditions  
2 followed protocol described by Hubka et al. (2018a). PCR product purification followed protocol of  
3 Réblová et al. (2016). Automated sequencing was performed at Macrogen Sequencing Service  
4 (Amsterdam, The Netherlands) using both terminal primers. The DNA sequences obtained in this study  
5 were deposited into the GenBank database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) under the accession numbers listed  
6 in Table 1.

7

## 8 **Phylogenetic analysis**

9 Alignments of the ITS, *gapdh*, *tubb* and *tefla* regions were performed using the FFT-NS-i option  
10 implemented with the MAFFT online service (Kato et al. 2017). The alignments were trimmed,  
11 concatenated and then analysed using maximum likelihood (ML) and Bayesian inference (BI) methods.  
12 Suitable partitioning schemes and substitution models (Bayesian information criterion) for the analyses  
13 were selected using a greedy strategy implemented in PartitionFinder 2 (Lanfear et al. 2017) with  
14 settings allowing introns, exons, codon positions and segments of the ITS region to be independent  
15 datasets. The optimal partitioning schemes for each analysed dataset along with basic alignment  
16 characteristics are listed in Table S3. The ML trees were constructed with IQ-TREE version 1.4.4  
17 (Nguyen et al. 2015) with nodal support determined by nonparametric bootstrapping (BS) with 1000  
18 replicates. The trees were rooted with *Trichophyton rubrum*. Bayesian posterior probabilities (PP) were  
19 calculated using MrBayes 3.2.6 (Ronquist et al. 2012). Optimal partitioning scheme and substitution  
20 models were selected as described above and are listed in Table S3. The analysis ran for 10<sup>7</sup> generations,  
21 two parallel runs with four chains each were used, every 1000<sup>th</sup> tree was retained, and the first 25 % of  
22 trees were discarded as burn-in. The convergence of the runs and effective sample sizes were checked  
23 in Tracer v1.6 (<http://tree.bio.ed.ac.uk/software/tracer>).

24 The modified complex indel coding (MCIC) algorithm implemented in SeqState version 1.25  
25 (Müller 2005) was used to code gaps. The TCS network method (Clement et al. 2000) was used to  
26 generate haplotype networks implemented in the program PopART (Leigh and Bryant 2015).

27

## 28 **Development of microsatellite markers**

29 Microsatellite motifs were identified in the available genomic sequence of *T. europaeum* CBS 112371  
30 = IHEM 20161 (<http://www.broadinstitute.org/>) using WebSat online software (Martins et al. 2009).  
31 The same program suggested optimal primers for the amplification of target loci. We selected di-, tri-,  
32 and tetranucleotide repeats based on the loci with the highest repeat numbers. Interrupted repeats as  
33 well as loci containing two or more repeat motifs within the fragments delimited by particular primer  
34 pairs were excluded. A pilot set of eight strains was used to evaluate microsatellite polymorphism for  
35 all candidate loci following the method of Schulke (2000). PCR conditions were as follows: one cycle  
36 at 95 °C for 1 min; 27 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, followed by eight cycles  
37 at 95 °C for 30 s, 53 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 10 min. A set of 24



1 loci exhibiting the highest level of polymorphism was selected from the 160 tested loci. The PCR  
2 products were screened for the presence of undesirable polymorphisms in the microsatellite flanking  
3 regions and the presence of polymorphisms in the microsatellite regions by sequencing. Emphasis was  
4 also placed on the selection of loci that were approximately uniformly distributed across the genome.  
5 Primer-primer interactions were checked before assembling multiplexes using Multiple Primer  
6 Analyzer (<http://www.thermoscientificbio.com/webtools/multipleprimer/>). The forward primers of ten  
7 selected loci were tagged with fluorescent dye and arranged into a single multiplex panel (Table 2). The  
8 reaction volume of 5  $\mu$ L for PCR contained 50 ng DNA, 0.5  $\mu$ L of the mixture of primers and 2.5  $\mu$ L  
9 of Multiplex PCR Master Mix (QIAGEN, Germany). The PCR conditions were chosen according to  
10 the manufacturer's recommendations. The PCR products (diluted in water 1:50) were mixed with 10  $\mu$ L  
11 of deionized formamide and 0.2  $\mu$ L of the GeneScan™ 600 LIZ size standard and denatured for 5 min  
12 at 95 °C, followed by analysis on an ABI 3100 Avant Genetic Analyzer.

13

#### 14 **Statistical analysis of microsatellite data**

15 The discriminatory power of these newly designed loci was calculated using Simpson's index of  
16 diversity as described previously (Hunter and Gaston 1988). A binary and allele data matrix was created  
17 using GeneMarker 1.51 software (SoftGenetics, LLC, State College, PA, USA) and used to estimate  
18 the similarities between individuals using Jaccard's similarity coefficient calculation in the program  
19 FAMD (Schlueter and Harris 2006). A neighbour-joining tree based on Jaccard's similarity coefficient  
20 matrix was constructed using the same software. Genetic distances were calculated from the same  
21 matrix and used for the construction of the NeighborNet network in the SplitsTree 4 program (Huson  
22 1998).

23 A Bayesian model-based clustering algorithm with a clustering number (K) = 1–10 was applied  
24 to the allele data matrix using the software STRUCTURE (Pritchard et al. 2000). Ten simulations were  
25 calculated at the [www.bioportal.uio.no](http://www.bioportal.uio.no) server (Lifeportal, University of Oslo) using the admixture  
26 model and  $10^6$  MCMC replicates;  $5 \times 10^8$  replicates were discarded as burn-in. The no-admixture model  
27 and uncorrelated allele frequencies were chosen for the analysis. The optimal clustering number (K)  
28 was estimated using  $\Delta K$  and similarity coefficients (Evanno et al. 2005), and both values were  
29 calculated using the script structure-sum (Ehrich 2006) in the R version 3.3.4 program (R\_Core\_Team  
30 2016).

31 The genetic variability within and between clusters was analysed for ten variable loci via  
32 analysis of molecular variance (AMOVA) in the Arlequin program (Schneider et al. 2000). The degree  
33 of gene flow among clusters was estimated using a pairwise fixation index ( $F_{ST}$ ) and a coefficient of  
34 genetic differentiation ( $G_{ST}$ ) calculated in Arlequin (Schneider et al. 2000) and POPGENE (Yeh et al.  
35 1999), respectively.

36 The degree of clonality or recombination within particular clusters was estimated by calculating  
37 the index of association ( $I_A$ ) in the program MultiLocus 1.3 (Agapow and Burt 2001), which is used for

1 measuring the linkage disequilibrium between alleles and is useful in inferring the occurrence of cryptic  
2 recombination in putatively asexual populations (Burt et al. 1996). Random mating is suggested if no  
3 linkage is detected between the alleles of different loci (randomly distributed alleles); in that case  $I_A$  is  
4 expected to be nearly zero or zero. We tested for significant deviation from 10 000 random multilocus  
5 permutations of genotypes under a random mating model.

6 To measure within-population diversity, Nei's genotype diversity ( $D_g$ ) was calculated based  
7 on frequencies of genetically distinct individuals, and Nei's gene diversity ( $D$ ) was calculated based on  
8 the frequencies of alleles at individual loci (Nei 1987, Kosman 2003). The effective number of  
9 genotypes ( $G_{\text{eff}}$ ) (Parker Jr 1979) was calculated based on the number of equally abundant genotypes  
10 required to reflect the value of a diversity measure. It was calculated to obtain diversity values  
11 comparable between the clusters. The degree of genetic divergence was investigated by rarity index of  
12 (DW index; frequency down-weighted marker values) (Schönswetter and Tribsch 2005). All mentioned  
13 population indexes ( $D_g$ ,  $D$ , DW,  $G_{\text{eff}}$ ) were calculated from the binary data matrix using script AFLPdat  
14 (Ehrich 2006) in R 3.0.2. Frequency histograms of pairwise differences between individuals were  
15 generated using the same program.

#### 17 **MAT locus determination and mating experiments**

18 A partial sequence of the MAT1-1-1 gene encoding the alpha box domain was amplified with the  
19 primers MF1 and MF5, and a partial sequence of the MAT1-2-1 gene encoding the high mobility group  
20 (HMG) domain was amplified with the primers Ab\_HMG\_F and Ab\_HMG\_R or TmHMG3S and  
21 TmHMG3R (Kano et al. 2012, Symoens et al. 2013, Kosanke et al. 2018). The PCR volume of 10  $\mu\text{L}$   
22 contained 25 ng of DNA, 0.15  $\mu\text{L}$  of both primers (25 pM  $\text{mL}^{-1}$ ), 0.15  $\mu\text{L}$  of My Taq Polymerase and  
23 2  $\mu\text{L}$  of buffer. The PCR conditions were described above. The PCR products were visualized in an  
24 electrophoretogram (1 % agarose gel with 0.5  $\mu\text{g mL}^{-1}$  ethidium bromide). Several PCR products of  
25 each MAT idiomorph were subjected to sequencing for the confirmation of product specificity.

#### 27 **Phenotypic studies**

28 *Macromorphology*. The morphology of the colonies on malt extract agar at 25 °C was documented in  
29 all strains. At least five strains from each species (if available) were subjected to a detailed analysis that  
30 involved macromorphology on MEA, potato dextrose agar (PDA, Himedia, Mumbai, India) and  
31 Sabouraud dextrose agar [SAB, Atlas (2010)] at 25, 30 and 37 °C (SAB). The macromorphology of the  
32 colonies was documented using an Olympus SZ61 or Canon EOS 500D binocular loupe (with Olympus  
33 Camedia C-5050 Zoom camera) or Canon EOS 500D. Colony colour determinations were made using  
34 the ISCC-NBS Centroid Colour Charts (Kelly 1964); <http://tx4.us/nbs/nbs-1.htm>.

35 *Micromorphology* was documented using an Olympus BX-51 microscope. Particular  
36 micromorphological characteristics were recorded at least 35 times for each isolate (at least five strains  
37 selected per species). The variance inflation factor (VIF) was assessed before performing the analysis

1 of variance to test the correlation between variables. Statistical differences in particular phenotypic  
2 characteristics were tested with one-way analysis of variance (ANOVA) followed by Tukey's honestly  
3 significant difference (HSD) test in program R version 3.3.4 (R\_Core\_Team 2016).

#### 5 **MALDI-TOF MS**

6 The cultivation of strains from the *T. benhamiae* clade (five strains from each species available) was  
7 performed in liquid cultivation medium for 22–24 h. The selected strains were prepared according to  
8 Schrödl et al. (2012) and analysed “as a blinde” by matrix-assisted laser desorption/ionization time-of-  
9 flight mass spectrometry (MALDI-TOF MS). In brief, for MALDI-TOF MS analysis, all samples were  
10 prepared using the liquid cultivation method and ethanol / formic acid extraction method. One milliliter  
11 of each over night culture was centrifuged for 2 min at about 10,000 g (= 980 cm/s g = 980 cm/s<sup>2</sup> 2.  
12 The supernatant was carefully removed and the fungal pellet was resuspended in 1 ml water, mixed  
13 thoroughly, and centrifuged for further 5 min at 10 000 × g. After removing the supernatant the pellet  
14 was resuspended in a mixture of 300 µL bidistilled water and 900 µL absolute ethanol. After  
15 centrifugation, the fungal cells were dried shortly and mixed thoroughly with 50 µL of 70 % formic  
16 acid and 50 µL pure acetonitrile, followed by centrifugation for 2 min at 10 000 × g. A volume of 1 µL  
17 supernatant was placed onto a MALDI target plate (Bruker Daltonik GmbH, Germany) and allowed to  
18 dry at room temperature. Eight MALDI target positions per strain were prepared in parallel. Each  
19 sample position (including one Bruker Bacterial Trest Standard position) was overlaid with 1 µL of  
20 matrix (HCCA portioned; Bruker Daltonik GmbH, Germany) and air dried at room temperature.  
21 MALDI-TOF MS measurement was conducted on a Microflex LT benchtop instrument operated by  
22 FlexControl software (Bruker Daltonik GmbH, Leipzig, Germany). Spectra were acquired in linear  
23 positive mode at a laser frequency of 200 Hz within a mass range from 2 000 to 20 000 Da by using the  
24 standard flexControl and AutoX methods. For each sampled spot up to three sum spectra were  
25 accumulated resulting in 24 MALDI spectra per strain. Finally, five spectra were selected for better  
26 spectra handling and visualization.

## 28 **RESULTS**

### 29 **Phylogeny of the *Trichophyton benhamiae* complex**

30 We assessed 340 combined ITS, *gadh*, *tubb* and *tef1-α* sequences from members of the *T. benhamiae*  
31 species complex (TBSC) in the phylogenetic analysis. The final alignment included 2371 characters,  
32 with 247 variable and 152 parsimony informative sites, and *Trichophyton rubrum* CBS 202.88 was used  
33 as the outgroup. The detailed alignment characteristics together with the partitioning schemes and  
34 substitution models are listed in Table S3. The isolation source and accession numbers for the DNA  
35 sequences are available in Table 1 and Table S1. The alignments were deposited in the Dryad Digital  
36 Repository: <https://doi.org/XXXXXX>.

1 Members of the TBSC were resolved into three major monophyletic clades in the best scoring  
2 multiple-gene ML tree shown in Figure 2, (single-gene trees are shown in Figure S2-S5).

3 The ***T. benhamiae* clade** contains anthropophilic *T. concentricum* (n = 3) and the European-  
4 American race of *T. benhamiae* (n = 318). The isolates of the European-American race do not form a  
5 monophyletic lineage and are paraphyletic with respect to *T. concentricum*. These strains are segregated  
6 into three major subclades: *T. benhamiae* s. str. and two newly proposed species, *T. japonicum* sp. nov.  
7 and *T. europaeum* sp. nov. Isolates of *T. benhamiae* s. str. originating mostly from Europe and North  
8 America, and they comprise both white and yellow phenotype strains. They form a monophyletic and  
9 fully supported (100 % bootstrap supports, bs/1.00 posterior probability, pp) subclade together with *T.*  
10 *concentricum*, which can be differentiated by only two unique substitutions in the ITS region and three  
11 in the *tef1-α* gene (the *tubb* and *gapdh* genes are identical).

12 Species from the *T. benhamiae* clade show a low level of intraspecific genetic variability. In  
13 total, there are only seven unique multilocus genotypes (MLST) among 318 isolates belonging to the  
14 *T. benhamiae* clade (Figure 3). Two MLST genotypes are present among *T. benhamiae* strains,  
15 represented by a single substitution in the *tef1-α* gene (Figure S4). Two MLST genotypes are present in  
16 *T. japonicum*, caused by a single substitution in the ITS1 region. *Trichophyton japonicum* can be  
17 differentiated from closely related *T. europaeum* by a single substitution in the ITS region and three  
18 conserved substitutions in the *gapdh* gene (Figure S2-S5). No intraspecific variability is detectable  
19 among the isolates of *T. europaeum*. The only exception is the isolate of “*T. europaeum*” IHEM 25139,  
20 which presents an abnormal ITS1 region sequence that contains 6 additional substitutions compared to  
21 the *T. europaeum* isolates. Some of these positions are critical for the differentiation of the *T.*  
22 *europaeum*/*T. japonicum* lineage from *T. benhamiae* s. str., suggesting that this strain could be hybrid  
23 between *T. benhamiae* clade species. The *gapdh* gene sequence of IHEM 25139 is typical of *T.*  
24 *europaeum*.

25 Both MAT gene idiomorphs were only detected among strains of *T. benhamiae*. *Trichophyton*  
26 *japonicum* and *T. concentricum* strains exhibited only the MAT1-1-1 idiomorph, while *T. europaeum*  
27 comprised strains characterized by the presence of the MAT1-2-1 idiomorph. Only “*T. europaeum*”  
28 strain IHEM 25139 showed MAT1-1-1 idiomorph.

29 The ***T. erinacei* clade** comprises three species: *T. erinacei*, an agent of mycoses in hedgehogs  
30 (genera *Erinaceus*, *Aterelix*); *T. verrucosum*, an agent of cattle ringworm; and *T. eriotrephon*, with  
31 poorly known ecological characteristics (Figure 2). All analyzed isolates of *T. erinacei* and *T.*  
32 *verrucosum* presented the MAT1-2-1 idiomorph, while *T. eriotrephon* exhibited only the MAT1-1-1  
33 idiomorph.

34 The ***T. bullosum* clade** contains three human isolates of the African race of *Arthroderma*  
35 *benhamiae*, and *T. bullosum* is a causal agent of dermatomycoses in horses and donkeys. Isolates of the  
36 African race apparently represent an independent taxonomic entity, and we propose the name *T.*

1 *africanum* for this species (Figure 2). Both MAT gene idiomorphs were detected in *T. africanum*, while  
2 *T. bullosum* isolates exhibited only the MAT1-1-1 idiomorph.

3

#### 4 **Analysis of the *T. benhamiae* clade with newly designed microsatellite markers**

5 A total of 160 microsatellite markers with di- or trinucleotide repeats and motifs longer than eleven  
6 repetitions were extracted from the available genome of *T. europaeum* CBS 112371 using WebSat  
7 software (Martins et al. 2009). The number of repeats was inferred by subtracting the known length of  
8 the flanking sequence from the total amplicon length. Only 24 regions contained the required repeat  
9 and showed length polymorphism in the microsatellite region and an absence of polymorphism in the  
10 flanking region. A total of ten markers with an even distribution in the genome and different lengths  
11 (for the purpose of multiplexing) were selected for the final analysis (Table 2). The Simpson's diversity  
12 index calculated for particular loci yielded values ranging from 0.34 (TC20 locus) to 0.59 (TAG16  
13 locus). The whole panel consisting of ten markers yielded a diversity index of 0.77 (Table S4).

14 This newly developed microsatellite typing scheme was applied to a total number of 318  
15 isolates belonging to the *T. benhamiae* clade. Forward primers of all loci were marked with fluorescent  
16 dye and arranged in a multiplex panel (Table 2). The highest number of alleles was found at the TAG16  
17 locus, followed by the CT21b locus. In contrast, the fewest alleles were found in at the AG18 (n = 5)  
18 and TC20 (n = 5) loci. The remaining loci included 7–9 alleles (Table S4). All loci were successfully  
19 amplified in all examined strains (null alleles were not found). The dependence of genotypic diversity  
20 on the number of loci showed that a sufficient number of markers was used to resolve the population  
21 structure of the *T. benhamiae* clade. It was apparent from the curves (Figure 4) that genetic diversity  
22 would not increase significantly with the addition of more markers.

23 The software STRUCTURE was used to determine how many groups were included in the  
24 dataset. The highest  $\Delta K$  value was observed at  $K = 6$ , and a much lower peak was present at  $K = 4$   
25 (Figure 5). The estimated population structure inferred from this analysis is shown in Figure 5. The  
26 analysis revealed a total of 41 genotypes among *T. benhamiae* clade isolates clustering into six clusters  
27 (C1-C6).

28 The distribution of the isolates into clusters was correlated with their geographic distribution  
29 and main primary hosts (Figure 6). The cluster C1 was found most abundantly in Europe and was  
30 associated with guinea pigs. These isolates are responsible for the current outbreak of infections in  
31 Central Europe and consists of yellow phenotype strains. We propose the name *T. benhamiae* var.  
32 *luteum* for this cluster. Clusters C2 and C3 comprised white phenotype strains from North America  
33 isolated mostly from dogs and characterized by highly variable microsatellite data (*T. benhamiae* var.  
34 *benhamiae*). Cluster C4 (*T. japonicum*) comprised the majority of strains from Japan analysed in this  
35 study and some European strains (rabbits, guinea pigs and human infections contracted from them).  
36 Cluster C5 (*T. europaeum*) comprised strains from Europe (infections mostly contracted from guinea  
37 pigs). The isolate IHEM 25139 was assigned to *T. europaeum* but its haplotype was intermediate

1 between *T. europaeum* and *T. japonicum* (alleles CT21 and CT21b were characteristic of *T. japonicum*,  
2 while the remaining 8 alleles were from *T. europaeum*). Cluster C6 was represented by three human  
3 isolates of *T. concentricum* from tropical regions.

4 The clustering based on the microsatellite data was correlated with MAT gene distribution and  
5 single-gene DNA data (*tubb* gene was excluded due lack of variability in *T. benhamiae* clade) (Figure 7,  
6 Figure S6). It is evident from the visualisation that clustering of isolates according to the single-gene  
7 genotype and MAT idiomorphs was in general agreement with microsatellite data and proposed species  
8 hypothesis. However, the clusters C1-C3 are not supported by any DNA locus sequences in study and  
9 are only distinguishable by microsatellites. *Trichophyton benhamiae* var. *luteum* (C1) was  
10 characterized by low variability of microsatellite data and exclusively consisted of isolates with MAT1-  
11 1-1 idiomorph. The isolates of *T. benhamiae* var. *benhamiae* cluster C2 were exclusively of the MAT1-  
12 2-1 idiomorph, while those of cluster C3 were exclusively of the MAT1-1-1 idiomorph. Despite obvious  
13 phenotypic and population genetic differences between *T. benhamiae* var. *benhamiae* and *T. benhamiae*  
14 var. *luteum*, these two varieties are not distinguishable by any of the DNA sequence markers used in  
15 this study. The only detected DNA sequence variant, represented by a single substitution in the *tef1-a*  
16 gene, did not correspond to the two varieties delimited by microsatellite markers. This substitution  
17 probably constitutes an incomplete lineage sorting phenomenon (Figure 7).

18

### 19 **Genetic diversity and population structure analysis of *T. benhamiae* clade**

20 Population characteristics were calculated from microsatellite data to test significance of clonal  
21 expansion versus recombination, and genetic diversity within clusters. Besides the inability to reproduce  
22 sexually due to missing opposite mating type in most of species, the clonality is indicated by the screwed  
23 distribution of pairwise differences between individuals (Figure 8). Consequently, all populations are  
24 genetically uniform which is evident from low value of Nei's gene diversity (*D*) (Table S5) that ranged  
25 from 0.02 in *T. benhamiae* var. *luteum* to 0.145 *T. benhamiae* var. *benhamiae*. The low Nei's genotype  
26 diversity ( $D_g = 0.35$ ) of *T. benhamiae* var. *luteum* compared to other taxa reflects the fact that the  
27 population consisted of several abundant clones (Table S5). Asexual reproduction prevails in all  
28 populations for long time which is supported by the low effective number of genotype ( $G_{\text{eff}}$ ) values that  
29 were significantly lower than observed number of genotypes (Table S5). The exception was *T.*  
30 *benhamiae* var. *benhamie* cluster C2 (Table S5). However, recombination in cluster C2 was not  
31 confirmed by calculation of index of association ( $I_A$ ) (Table S5), possibly due to low number of samples  
32 available. The recombination was not rejected only in *T. europaeum* population according according to  
33  $I_A$  on significance level  $p < 0.05$  ( $I_A = 0.24$ ,  $p < 0.0042$ ) (Figure 9, Table S5).

34 To test cluster-specific differences, AMOVA was performed on the microsatellite data. The  
35 diversity between six clusters contributed to a total variability of 68.1 %, while the diversity within  
36 clusters contributed to only 31.9 % ( $p < 0.0001$ ). Thus, there is a low level of genetic information  
37 exchange between clusters, reflected in a high number of fixed alleles ( $F_{\text{ST}} = 0.89$ ,  $G_{\text{ST}} = 0.75$ ,

1 p<0.0001). Low gene flow levels between *T. benhamiae* var. *luteum*, *T. japonicum* and *T. europaeum*  
2 demonstrated by these indices could be explained by reproductive isolation despite overlapping hosts  
3 (e.g. guinea pigs) and geographic distributions. This could be caused by pre- or postzygotic reproductive  
4 barriers, or absence of terrestrial reservoir for sexual reproduction.

5 *Trichophyton concentricum* and *T. benhamiae* var. *benhamiae* cluster C3 shared the greatest  
6 number of alleles in common ( $F_{ST} = 0.451$ ,  $G_{ST} = 0.49$ ). The lowest number of shared alleles was found  
7 between *T. benhamiae* var. *luteum* and all other clusters ( $F_{ST} = 0.90$ – $0.95$ ; Table S6). The strongly fixed  
8 set of unique alleles in *T. benhamiae* var. *luteum* indicates low or no gene flow between this cluster and  
9 the remaining clusters. Relatively low DW index value ( $DW = 0.06$ ; Table S5) indicate recent origin of  
10 *T. benhamiae* var. *luteum*. On the other hand, high DW values in other taxa indicate long-term isolation  
11 due to accumulation of unique alleles (Table S5).

### 13 **Phenotypic studies**

14 Initially, the phenotype of all isolates was recorded on malt extract agar (MEA). It was observed that  
15 the morphotypes within the *T. benhamiae* clade generally corresponded to the clusters delimited by  
16 microsatellite analysis. Notable exceptions were the strains showing signs of degeneration (poorly  
17 sporulating, white, cottony colonies usually producing no pigments). Such a phenotype is commonly  
18 described in dermatophytes and indicates degeneration, usually caused by long-term strain passaging  
19 and preservation. These strains were excluded from further phenotype analyses. At least five strains (if  
20 available) from each group were selected, and their phenotypes were analysed on three cultivation  
21 media (Figure 10). Growth rates were recorded at three temperatures (Figure 11), and micromorphology  
22 was measured on MEA (Figure 12). Cultivation on MEA and potato dextrose agar (PDA) promoted  
23 sporulation and pigment production most effectively.

24 Among the taxa from the *T. benhamiae* clade, the strains of *T. concentricum* and *T. benhamiae*  
25 var. *luteum* were characterized by the slowest growth on all media and at all tested temperatures (Figure  
26 11). No sporulation was observed in the *T. concentricum* strains examined in this study. Overall, poor  
27 sporulation, the production of intense yellow pigmentation as the colony reverse colour and the absence  
28 of macroconidia and spiral hyphae were characteristic of *T. benhamiae* var. *luteum* (yellow phenotype  
29 strains of *T. benhamiae*). All three remaining species from the *T. benhamiae* clade produced both micro-  
30 and macroconidia and whitish colonies, usually with a brownish, red-brown or red colony reverse colour  
31 (white phenotype strains of *T. benhamiae*). *Trichophyton benhamiae* var. *benhamiae* grew more rapidly  
32 at 25 °C than the other species from this clade (Figure 11) and exhibited larger microconidia on average  
33 (Figure 12). The obverse colony colour was whitish or showed a brownish tint, and red-brown  
34 pigmentation on the reverse side was commonly arranged into sectors (Figure 10). The growth  
35 parameters and micromorphology of *T. japonicum* and *T. europaeum* were very similar (Figure 11,  
36 Figure 12), and all strains extensively sporulated.

1 Phylogenetically distant *T. africanum* (formerly African race of *T. benhamiae*) was  
2 characterized by relatively long microconidia (comparable to those of *T. benhamiae* var. *benhamiae*)  
3 growing on unbranched or loosely branched conidiophores. Compared to *T. africanum*, the  
4 conidiophores of *T. benhamiae* clade members were either poorly differentiated from vegetative hyphae  
5 (conidia sessile on the hyphae) or short with many lateral branches under the top (branched in a  
6 pyramidal pattern, grape-like). A more detailed differential diagnosis of particular species with their  
7 relatives is included in the Notes in the Taxonomy section.

8 The ANOVA was performed on microconidia width, length and growth rates (MEA, SAB,  
9 PDA at 25, 30 and 37 °C), followed by a post hoc analysis using Tukey's HSD pairwise comparisons  
10 based on the mean values for each strain and a confidence interval of 0.95. All growth rate variables  
11 and conidium size variables were strongly correlated. The variables from the two groups can therefore  
12 be used interchangeably (Figure 13). The analysis showed that there were statistically significant  
13 differences between *T. benhamiae* clade species according to any combination of characteristics,  
14 including conidia size and growth rates ( $p < 0.001$ ). Furthermore, growth rates measured at 25 °C on  
15 MEA or PDA can be used independently to distinguish the majority of species ( $p < 0.001$ ) (Figure 13,  
16 Figure S7, Table S7). Variables such as microconidium length (Table S8) and width (Table S9) can also  
17 be used independently to distinguish particular species, except for *T. japonicum* and *T. benhamiae* var.  
18 *luteum*, which cannot be differentiated at the specified significance level.

#### 19 20 **MALDI-TOF mass spectrometry**

21 Representative isolates of each species from the *T. benhamiae* clade were analysed using MALDI-TOF  
22 mass spectrometry; *T. africanum* isolates were also included for comparison (Figure 14). All samples  
23 could be measured very well and delivered high quality (peak rich) MALDI spectra. In the mass range  
24 between approximately 5900-6200 m/z (as an representative example), the MALDI-TOF mass spectra  
25 were very similar both between and within all groups, and differentiation of the groups was not possible  
26 within this range. In contrast to this high similarity, several specific peaks could be found for all  
27 analysed taxa the entire mass range of approximately 4000 to 12,000 m/z (Figure 14). *Trichophyton*  
28 *africanum* significantly differed from all of the samples in many peaks in its spectrum (Figure 14A).  
29 *Trichophyton benhamiae* var. *luteum* and *T. benhamiae* var. *benhamiae* shared peaks at 7150 and 7745  
30 m/z in their mass spectra but different peaks at 4112 and 4680 m/z, which are typical of var. *luteum*,  
31 and 6515 and 6530 m/z, which are typical of var. *benhamiae* (Figure 14C-D). Both mentioned species  
32 differ from *T. europaeum* and *T. japonicum* in the absence of a peak at 7150 m/z (data not shown).  
33 *Trichophyton europaeum* differed from *T. japonicum* in the presence of a peak at 7745 m/z and the  
34 absence of a peak at 7715 m/z (Figure 14B). *Trichophyton concentricum* differed from both *T. benhamiae*  
35 varieties in its peaks at 4770, 6435 and 7145 m/z (Figure 14D) and also differed from the rest of the  
36 samples in several peaks. To prove the general applicability of the here presented MALDI peaks more



1 strains of the mentioned species / varieties should be analyzed in the future and incorporated into the  
2 presented MALDI-based differentiation model.

3

#### 4 **TAXONOMY**

##### 5 ***Trichophyton benhamiae* clade**

6

7 ***Trichophyton benhamiae*** (Ajello & S.L. Cheng) Y. Gräser & de Hoog [Index Fungorum 356: 2. 2018]  
8 **var. *benhamiae*** (automatically generated; Art. 26.3 [Turland et al. (2018)]) — MycoBank **XXXX**;  
9 Figure 15

10

11 *Vegetative hyphae* smooth, septate, hyaline, 1.5–4 µm diam (mean ± sd; 2.5 ± 0.7). *Conidiophores*  
12 poorly differentiated from vegetative hyphae, mostly unbranched, conidia sessile or born on short lateral  
13 branches; pyramidally branches conidiophores less common and with sparse branching. *Microconidia*  
14 abundant, pyriform to clavate, truncate, 2.5–6 (3.8 ± 0.5) × 1.6–3.5 (2.6 ± 0.4) µm. *Macroconidia* sparse  
15 to abundant, cylindrical or elongated fusiform, with pointed or rounded ends, easily disintegrate into  
16 fragments with truncate ends, developing intercalary or terminally on vegetative hyphae, frequently  
17 released with short to long mycelial fragments at one or both ends, predominantly 3–10-septate (median  
18 8), 23–82 (59.2 ± 15.5) × 4.5–7.5 (6.1 ± 0.8) µm. *Chlamydospores* present. *Spiral hyphae* absent or  
19 rare. Heterothallic. *Sexual state* *fide* Ajello & Cheng (1967) and Čmoková (2015): *Cleistothecia* white  
20 to yellowish-white, covered with dichotomously branched peridial hyphae and spiral appendages.  
21 *Peridial hyphae* composed of asymmetrical peridial cells, dumb-bell shaped, echinulate, 8.5–10.5 (9.1  
22 ± 1.8) µm in length, 2.5–4.5 (2.8 ± 0.7) µm in width at enlarged ends, internode width 2–4 µm (2.4 ±  
23 1.2); intercalary conidia sparse, cylindrical or barrel-shaped. *Asci* globose, eight-spored, *ascospores*  
24 ovate, hyaline to pale yellow, longer dimension up to 3 µm, shorter dimension up to 2 µm.

25

26 *Culture characteristics* — (Colonies in 7 d at 25 °C) Colonies on SAB 28–34 mm diam (ø = 32 mm),  
27 White (#F2F3F4), velvety to powdery, centrally raised, radially furrowed in some strains, edge diffuse,  
28 reverse Pale Orange Yellow (#FAD6A5) to Light Orange Yellow (#FBC97F) in the marginal part,  
29 Vivid Orange (#F38400) to Deep Brown (#593319) in the center. Colonies on MEA 30–35 mm diam  
30 (ø = 34 mm), velvety to granular, Pale Yellow-gray (#C7ADA3) to Light Yellow (#FAD6A5),  
31 umbonate, edge diffuse, reverse Pale Orange Yellow (#FAD6A5) to Brilliant Orange Yellow  
32 (#FFC14F), red pigment produced in sectors by some strains - Deep Reddish Orange (#AA381E).  
33 Colonies on PDA 27–32 mm diam (ø = 30 mm), White (#F2F3F4) to Light Yellow (#FAD6A5), velvety  
34 to granular, centrally raised, occasionally with filamentous sectors, reverse Pale Orange Yellow  
35 (#FAD6A5) to Brilliant Orange Yellow (#FFC14F), red pigment produced in sectors by some strains -  
36 Deep Reddish Orange pigment (#AA381E). Colonies in 7 d at 30 °C grow faster than at 25 °C: SAB  
37 37–45 mm diam (ø = 39 mm); PDA 35–43 mm diam (ø = 37 mm); MEA 8–43 mm diam (ø = 40 mm).

1 Colonies at 37 °C in 7 d: SAB 27–39 mm diam ( $\varnothing$  = 30 mm); PDA 30–35 mm diam ( $\varnothing$  = 34 mm); MEA  
2 30–35 mm diam ( $\varnothing$  = 33 mm).

3

4 *Specimens examined.* USA, Missouri, human, Ajello [epitype designated here MBTXXXX, PRM  
5 944659, a dried culture derived from strain IHEM 4710, culture ex-type IHEM 4710 (= CBS 623.66 =  
6 ATCC 16781 = CABIM 124768 = CDC X-797 = CECT 2892 = IMI 124768 = IP 1064.74 = NCPF  
7 0410 = RV 23303 = UAMH 2822]. USA, Urbana, dog, 2009 (USA 3208). USA, Urbana, dog, 2006 (USA  
8 3209); *ibid.*, USA 3216. USA, Urbana, cat, 2006 (USA 3220). USA, Urbana, dog, 2007 (USA 3329).  
9 USA, Urbana, dog, 2010 (USA 3350); *ibid.*, USA 3355; *ibid.*, USA 3356. USA, Urbana, chinchilla, 2011  
10 (USA 3360). USA, Urbana, dog, 2011 (USA 3361). USA, Urbana, unknown source, 1991 (USA 3368).  
11 USA, Urbana, unknown source, 1989 (USA 3369). USA, Urbana, unknown source, 1997 (USA 3370).  
12 USA, Urbana, unknown source, 2001 (USA 3371). USA, Urbana, unknown source, 1996 (USA 3376).  
13 USA, Urbana, unknown source, 1995 (USA 3378). IN-VITRO, monoascospore isolate, 1970, M. Takashio  
14 [IHEM 3287 = RV 26678; isolate from cross between IHEM 24908 (ex dog, USA)  $\times$  IHEM 4710 (ex  
15 human, USA)]. IN-VITRO, monoascospore isolate, 1970, M. Takashio [IHEM 3288 = BER 1464 = DSM  
16 6916 = JS 83-006 = RV 26680 = SM 0104 = VUT 77012 = CCRC 31780 = IAM 12705 = JCM 1886;  
17 isolate from cross between IHEM 24908 (ex dog, USA)  $\times$  IHEM 4710 (ex human, USA)].

18

19 *Typification* — Ajello & Cheng (1967) designated the specimen NCDC B765d as a holotype of *T.*  
20 *benhamiae*, and a dried culture with ascomata was generated by crossing the isolates TM-20 (= ATCC  
21 16781 = IHEM 4710 = CBS 623.66 = CABIM 124768 = CDC X-797 = CECT 2892 = IMI 124768 =  
22 IP 1064.74 = NCPF 0410 = RV 23303 = UAMH 2822; ex human; MAT1-2-1)  $\times$  TM-17 (= ATCC  
23 16782 = CBS 624.66 = IHEM 24908 = RV 23302 = CDC X-798 = CECT 2893 = IMI 124769 = NCPF  
24 411 = UAMH 2823; ex dog; MAT1-1-1). Although this specimen exhibits both sexual and asexual  
25 morphs in its life cycle, it is not suitable for the purposes of the recent taxonomy for several reasons.  
26 First, it is not clear which of the two cultures contained within the type should be considered the ex-  
27 holotype culture. Additionally, interspecific hybrids can be induced by crossing opposite mating type  
28 strains of unrelated species *in vitro* as shown in previous studies on dermatophytes (Kawasaki et al.  
29 2009, Anzawa et al. 2010, Kawasaki et al. 2010, Kawasaki et al. 2011), and the deposition of a resultant  
30 ‘hybrid’ type could lead to ambiguities. Because it is not possible to recognize which portion of the  
31 holotype belongs to a particular isolate, we designated an epitype PRM 944659 (dried culture) derived  
32 from the IHEM 4710 (= CBS 623.66 = ATCC 16781 = CABIM 124768 = CDC X-797 = CECT 2892  
33 = IMI 124768 = IP 1064.74 = NCPF 0410 = RV 23303 = UAMH 2822) .

34

35 *Distribution and ecology* — *Trichophyton benhamiae* var. *benhamiae* is a zoophilic dermatophyte, and  
36 isolates examined in this study originated from dogs (n = 8), cats (isolate USA 3220), chinchillas (isolate  
37 USA 3360) and unknown hosts (n = 6). Previously reported cases of human infections were probably

1 transmitted from animals (Ajello and Cheng 1967). Another important host of this pathogen is probably  
2 the North American porcupine (*Erethizon dorsatum*) (Takahashi et al. 2008, Needle et al. 2019), a close  
3 relative of the guinea pig (*Cavia porcellus*). Isolates from the North American porcupine exhibited ITS  
4 rDNA identical to that of *T. benhamiae*, and their morphology showed characteristics typical of *T.*  
5 *benhamiae* var. *benhamiae* (Takahashi et al. 2008, Needle et al. 2019). All strains examined here were  
6 collected in North America (the in vitro-derived isolates were also based on strains of American origin).  
7 A recently reported a Chinese case of tinea faciei, likely contracted from fox, that was probably also  
8 caused by *T. benhamiae* var. *benhamiae* based on the ITS sequence and morphology of the isolate (Tan  
9 et al. 2020).

10

11 Notes — The macromorphology of *T. benhamiae* var. *benhamiae* most closely resembles those of *T.*  
12 *europaeum*, *T. japonicum* and *T. mentagrophytes* in the production of a red-brown pigment on reverse  
13 side of colonies and abundant microconidia. It differs from *T. europaeum* and *T. japonicum* in its host  
14 spectrum and higher growth rates, especially on MEA and PDA at 25 °C (Figure 11). Macroconidia of  
15 *T. benhamiae* var. *benhamiae* are usually more abundantly produced compared to *T. europaeum* and *T.*  
16 *japonicum*, and they are most frequently cylindrical or elongated fusiform with terminal fragments of  
17 vegetative hyphae. Closely related *T. concentricum* differs significantly in its ecology. It is an  
18 anthropophilic species occurring in tropical regions, grows very slowly, produces cerebriform colonies  
19 without red-brown pigment on the colony reverse and usually does not sporulate. *Trichophyton*  
20 *benhamiae* var. *luteum* is also strikingly different in its host spectrum (mostly guinea pigs), distribution  
21 (mainly Europe) and morphology (slow growth, yellow pigmentation, relatively poor sporulation,  
22 absence of macroconidia). *Trichophyton benhamiae* var. *benhamiae* does not produce intense yellow  
23 pigment on SAB supplemented with chloramphenicol and cycloheximide and MEA, in contrast to *T.*  
24 *benhamiae* var. *luteum*. The ratio of MAT1-1-1 and MAT1-2-1 strains was 14:5.

25

26 ***Trichophyton benhamiae*** (Ajello & S.L. Cheng) Y. Gräser & de Hoog [Index Fungorum 356: 2. 2018]  
27 var. ***luteum*** Cmokova & Hubka, var. nov. — MycoBank XXXX; Figure 16

28

29 *Etymology.* Refers to the bright yellow colonies produced especially on SAB with  
30 chloramphenicol and cycloheximide and MEA.

31

32 *Vegetative hyphae* smooth, septate, hyaline, 1–3.5 µm diam (mean ± sd: 1.9 ± 0.5). *Conidiophores*  
33 branched in a pyramidal (grape-like) pattern, sometimes poorly differentiated from vegetative hyphae,  
34 unbranched or poorly branched, conidia sessile or born on short lateral branches. *Microconidia* sparse  
35 to abundant, pyriform, less commonly clavate, 2.5–4.9 (3.2 ± 0.4) × 1.5–3.4 (2.1 ± 0.3) µm.  
36 *Macroconidia* not observed in any of the isolates examined. *Chlamydospores* were not observed. *Spiral*  
37 *hyphae* not observed. *Sexual morph* unknown.

1  
2 *Culture characteristics* — (Colonies in 7 d at 25 °C) colonies on SAB 10–20 mm diam ( $\varnothing = 13$  mm),  
3 White (#F2F3F4) to Yellowish White (#F0EAD6), velvety, flat with radially furrowed center, edge  
4 filliform, reverse Vivid Yellow (#F3C300). Colonies on MEA 6–17 mm diam ( $\varnothing = 12$  mm), Pale Yellow  
5 (#F3E5AB), filamentous, flat, edge filliform, reverse Light Yellow (#F8DE7E) to Vivid Yellow  
6 (#F3C300). Colonies on PDA 9–17 mm diam ( $\varnothing = 13$  mm), Light Yellow (#F8DE7E) to Pale Yellow  
7 (#F3E5AB), velvety, flat, radially furrowed, edge filliform, reverse Brilliant Orange Yellow (#FFC14F)  
8 to Vivid Yellow (#F3C300). Colonies at 30 °C in 7 d: SAB 15–26 mm diam ( $\varnothing = 21$  mm); PDA 18–22  
9 mm diam ( $\varnothing = 21$  mm); MEA 21–22 mm diam ( $\varnothing = 22$  mm). Colonies at 37 °C in 7 d: SAB 15–20 mm  
10 diam ( $\varnothing = 18$  mm); PDA 10–17 mm diam ( $\varnothing = 12$  mm); MEA 11–13 mm diam ( $\varnothing = 11$  mm).

11  
12 *Specimens examined*. SWITZERLAND, Lausanne, University Hospital Vaudois, dermatophytosis in  
13 human, arm skin (tinea corporis), 2009, M. Monod (PRM 944414, holotype, dried culture; PRM  
14 944415, isotype; culture ex-type IHEM 25068). JAPAN, common degu, 2012 (NUBS 13001).  
15 SWITZERLAND, Lausanne, University Hospital Vaudois, human skin, 2009, M. Monod (IHEM 25066).  
16 CZECHIA, Prague, guinea pigs (*Cavia porcellus*), 2014, J. Koubová (KOUB 23); *ibid.*, KOUB 51; *ibid.*,  
17 KOUB 77. GERMANY, Berlin, dermatophytosis in human, 2010 (BER 24); *ibid.*, BER 211; *ibid.*, BER  
18 212; *ibid.*, BER 213. CZECHIA, České Budějovice, dermatophytosis in human, 2012 (D126); *ibid.*,  
19 D295; *ibid.*, D375; *ibid.*, D417; *ibid.*, D521. GERMANY, Mölbis, dermatophytosis in human, 2015 (DE  
20 200156); *ibid.*, DE 200351; *ibid.*, DE 200465. BELGIUM, Brussels, dermatophytosis in human, 2012  
21 (IHEM 25744); *ibid.*, IHEM 25743; *ibid.*, IHEM 25742; *ibid.*, IHEM 25466; *ibid.*, IHEM 25745.  
22 CZECHIA, Prague, dermatophytosis in human, 2012 (CCF 4849); *ibid.*, CCF 4850; *ibid.*, CCF 4851;  
23 *ibid.*, CCF 4852. All 236 strains examined in this study are listed in Table S1.

24  
25 *Distribution and ecology* — *Trichophyton benhamiae* var. *luteum* is a zoophilic species with the guinea  
26 pig as the main host (Hubka et al. 2018c). It is widely distributed in Europe, but it has also been detected  
27 in common degu (*Octodon degus*) in Japan (Hiruma et al. 2015) and was recently isolated from human  
28 dermatophytosis in Brazil (de Freitas et al. 2019, Grisólia 2019).

29  
30 *Notes* — The macromorphology of *T. benhamiae* var. *luteum* resembles that of *Microsporum canis* in  
31 the production of intense yellow pigmentation as the colony reverse colour. However, *M. canis* usually  
32 produces abundant spindle-shaped macroconidia, which are absent in *T. benhamiae* var. *luteum*. The  
33 differentiation of sterile *M. canis* isolates may be more difficult but is possible according to its higher  
34 growth parameter values. In addition, these species differ in their main hosts, which are cats and dogs  
35 in *M. canis* and guinea pigs in *T. benhamiae* var. *luteum*. The closely related anthropophilic species *T.*  
36 *concentricum* differs in its ecology, colony characteristics (no yellow pigment produced) and

1 microscopic characteristics (usually no sporulation). Other taxa from the *T. benhamiae* clade differ in  
2 showing higher growth rates (Figure 11), the production of red/brown pigments and the production of  
3 macroconidia, which are absent in *T. benhamiae* var. *luteum*. In addition to these differences, *T.*  
4 *benhamiae* var. *luteum* can be clearly distinguished from *T. benhamiae* var. *benhamiae* and other  
5 species in the *T. benhamiae* clade by microsatellite data (Figures 5–6) and MALDI-TOF MS spectra  
6 (Figure 14).

7         The European strains of *T. benhamiae* var. *luteum* (n = 236) examined here were predominantly  
8 obtained from humans (~72 % from females and ~28 % from males, median age 12 years) who mostly  
9 reported contact with guinea pigs; the remaining strains were recovered from animals (guinea pigs and  
10 common degu) (Table S1). The human infections mostly manifested as highly inflammatory tinea  
11 corporis, tinea faciei and tinea capitis (Fig. 17). By contrast, infected animals were mostly symptomless.  
12 Symptomatic guinea pigs usually showed localized lesions with scaling and crusting or alopecia located  
13 predominantly on the head, less frequently on the other body parts (Fig. 17). Green fluorescence of  
14 infected tissues may be observed under Wood's light in some strains, similar to *M. canis* (Skořepová et  
15 al. 2014). Only the MAT1-1-1 idiomorph was detected in the *T. benhamiae* var. *luteum* isolates  
16 examined here.

17

18 ***Trichophyton concentricum*** R. Blanch., *Traité de Pathologie Générale* 2: 916. 1896 — Figure 18

19

20 *Vegetative hyphae* smooth, septate, frequently inflated, occasionally with knob-like terminations, often  
21 proliferating in a zigzag pattern, hyaline, 1.5–4 µm diam (mean ± sd; 2.7 ± 0.7). *Chlamydoconidia*  
22 common, usually globose or ovate, intercalary, terminal or in short chains. *Conidiophores*, *conidia*,  
23 *pectinate hyphae* and *favic chandeliers* were not observed among the examined strains. *Sexual morph*  
24 unknown.

25

26 *Culture characteristics* — (Colonies in 7 d at 25 °C) Colonies on SAB 6–16 mm diam (∅ = 11 mm),  
27 Pale Orange Yellow (#FAD6A5) to Pale Yellowish Pink (#ECD5C5), membranous to slightly velvety,  
28 raised, umbonate or cerebriform, deeply furrowed, edge filiform or lobate, reverse Light Orange Yellow  
29 (#FBC97F). Colonies on MEA 9–16 mm diam (∅ = 15 mm), Pale Orange Yellow (#FAD6A5) to Pale  
30 Yellowish Pink (#ECD5C5), membranous to slightly velvety, umbonate, edge filiform, reverse Light  
31 Orange Yellow (#FBC97F) to Brilliant Orange Yellow (#FFC14F), Vivid Yellow (#F3C300) in narrow  
32 centre. Colonies on PDA 5–12 mm diam (∅ = 11 mm), Pale Orange Yellow (#FAD6A5) to Pale  
33 Yellowish Pink (#ECD5C5), membranous, raised, deeply furrowed to cerebriform, edge irregular to  
34 lobate, reverse Light Orange Yellow (#FBC97F) to Brilliant Orange Yellow (#FFC14F), Vivid Yellow  
35 (#F3C300) in narrow centre. Colonies at 30 °C in 7 d: SAB 8–20 mm diam (∅ = 16 mm); MEA 8–15  
36 mm diam (∅ = 13 mm); PDA 10–14 mm diam (∅ = 11 mm). Colonies at 37 °C in 7 d: SAB 5–14 mm  
37 diam (∅ = 10 mm); MEA 5–13 mm diam (∅ = 10 mm); PDA 5–13 mm diam (∅ = 9 mm).

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*Specimens examined.* POLYNESIA, human, 1926, A. Castellani (ex-neotype strain CBS 196.26 = IFO 5972). FIJI, human skin, 1963 (CCF 5303 = IHEM 13435 = RV 30442). INDONESIA, Manado, human, arm and trunk skin, 1990, W. Warow (CCF 5302 = IHEM 5470)

*Distribution and ecology* — *Trichophyton concentricum* is an anthropophilic species distributed in Oceania, Southeast Asia, and Central and South America. It is a cause of tinea imbricata (tokelau) usually affecting rural indigenous populations. The clinical manifestation is very characteristic and gives human skin ornate appearance due to the presence of concentric squamous plaques (Bonifaz et al. 2004, Pihet et al. 2008, Bonifaz and Vazquez-Gonzalez 2011).

*Notes* — The morphology of *T. concentricum* resembles those of the slow-growing species *T. verrucosum*, *T. bullosum* (for differentiation see *T. bullosum* description) and *T. schoenleinii*. Closely related species from the *T. benhamiae* clade are easily distinguished from *T. concentricum* by higher growth rates (Figure 11) and relatively abundant sporulation. Differentiation from these species is usually not problematic in practice due to the different host spectra and geographic distributions of these species. Only the MAT1-1-1 idiomorph was detected in the *T. concentricum* isolates examined here; in contrast, isolates of *T. verrucosum* and *T. schoenleinii* exclusively show the MAT1-2-1 idiomorph (Kano et al. 2014, Kosanke et al. 2018).

*Trichophyton concentricum* usually grows as a sterile mycelium in culture; however, the production of clavate microconidia and smooth-walled macroconidia has been observed by some authors (Rippon 1988, Pihet et al. 2008), while favic chandeliers and pectinate hyphae (“antler” tips) are more frequently reported (Dvořák and Otčenášek 1969, Bonifaz et al. 2004). We did not observe these structures in any of the isolates examined.

***Trichophyton europaeum*** Cmokova & Hubka, *sp. nov.* — MycoBank **XXXXX**; Figure 19

*Etymology.* Refers to the origin of the examined strains.

*Vegetative hyphae* smooth, septate, hyaline, 1–3 µm diam (mean ± sd: 1.9 ± 0.3). *Conidiophores* branched in a pyramidal (grape-like) pattern or poorly differentiated from the hyphae and represented by conidiogenous hyphae with sparse to numerous short lateral branches. *Microconidia* abundant, sessile on lateral or terminal branches, pyriform to clavate, 2.5–3.9 (3 ± 0.3) × 1.5–2.8 (2.1 ± 0.2) µm. *Macroconidia* rare to sparse, born terminally on hyphae, rare to sparse, usually consisting of 2–7 cells (median = 4) with an unequal diameter, 45–76 (51.2 ± 7.3) × 3–10.5 (5 ± 1.3) µm, elongated, clavate, less frequently fusiform, with a tapering rounded apex and truncate end, cylindrical fragments of macroconidia common, macroconidia consisting of irregular and bloated cells common.

1 *Chlamydospores* present. *Spiral hyphae* absent to rare in 14-d-old cultures, usually consisting of one to  
2 several coils. *Sexual morph* unknown, pseudo-ascomata are formed by some isolates after prolonged  
3 incubation.

4

5 *Culture characteristics* — (Colonies in 7 d at 25 °C) Colonies on SAB 24–29 mm diam ( $\varnothing = 25$  mm),  
6 White (#F2F3F4), velvety to floccose, flat, in some strains with radially wrinkled or elevated center,  
7 edge filiform, diffuse or entire, reverse Brilliant Yellow (#FADA5E), to Deep Orange Yellow  
8 (#C98500). Colonies on MEA 20–30 mm diam ( $\varnothing = 26$  mm), White (#F2F3F4) to Light Yellow  
9 (#F8DE7E), velvety, floccose to coarsely granular, flat with an umbonate center, edge entire to diffuse,  
10 reverse in shades of brown [Strong Orange Yellow (EAA221) to Deep Orange (#BE6516)] or red [Vivid  
11 Reddish Orange (#E25822) to Vivid Red (#BE0032)]. Colonies on PDA 19–23 mm diam ( $\varnothing = 21$  mm),  
12 White (#F2F3F4) to Light Yellow (#F8DE7E), velvety, floccose to coarsely granular, flat with an  
13 umbonate center, edge irregular, lobate dendritic, reverse yellow (#F3C300) in the marginal part, Strong  
14 Orange Yellow (#EAA221) to Deep Orange (#F38400) in the center. Colonies at 30 °C in 7 d: SAB  
15 32–37 mm diam ( $\varnothing = 35$  mm); PDA 29–31 mm diam ( $\varnothing = 30$  mm); MEA 32–39 mm diam ( $\varnothing = 36$  mm).  
16 Colonies at 37 °C in 7 d: SAB 23–31 mm diam ( $\varnothing = 28$  mm); PDA 24–31 mm diam ( $\varnothing = 29$  mm); MEA  
17 20–30 mm diam ( $\varnothing = 27$  mm).

18

19 *Specimens examined*. SWITZERLAND, Lausanne, guinea pig (*Cavia porcellus*), 2008, M. Monod (PRM  
20 944419, holotype, dried culture; ex-holotype culture IHEM 22725). FRANCE, Lyon, guinea pig, 1963  
21 (IHEM 25139 = CBS 806.72 = RV 14387). SWITZERLAND, Lausanne, human dermatophytosis (contact  
22 with guinea pig), 2002, M. Monod (IHEM 20159 = CBS 112370); *ibid.*, IHEM 25062. SWITZERLAND,  
23 Lausanne, human dermatophytosis (contact with guinea pig), 2007, M. Monod (IHEM 25064).  
24 SWITZERLAND, Lausanne, tinea corporis (contact with guinea pig), 2010, M. Monod (IHEM 25075).  
25 SWITZERLAND, Lausanne, tinea faciei, 2011, Monod (HEM 25076). SWITZERLAND, Lausanne, guinea  
26 pig, 2002, M. Monod (IHEM 22723). Czechia, Ostrava, 5-years girl, 2012, S. Dobiášová (CCF 4917).  
27 Czechia, Prague, human dermatophytosis (tinea faciei), 2012, M. Skořepová (CCF 4848). CZECHIA,  
28 Bylany, dermatophytosis in human (tinea corporis), M. Skořepová (CCF 4853). All 40 strains of *T.*  
29 *europaeum* examined in this study are listed in Table S1.

30

31 *Distribution and ecology* — *Trichophyton europaeum* is a zoophilic species that is widely distributed  
32 in guinea pigs in Europe (Fumeaux et al. 2004, Fréalle et al. 2007, Symoens et al. 2013) but is currently  
33 less prevalent than *T. benhamiae* var. *luteum*. The species has also been reported from guinea pigs in  
34 Japan (Takeda et al. 2012) and human dermatophytosis in Iran (Rezaei-Matehkolaei et al. 2016).  
35 Dermatophytosis in horses reported in Egypt is an unusual finding (Tartor et al. 2016).

1 The European strains of *T. europaeum* (n = 41) examined here were predominantly obtained  
2 from humans (~80 % from females and ~20 % from males, median age 12 years) who mostly reported  
3 contact with guinea pigs (66 %), and the remaining strains were recovered from animals (guinea pigs,  
4 24 %) or dogs (Table S1). The infections mostly manifested as tinea corporis (79 %) and tinea faciei  
5 (21 %). Only the MAT1-2-1 idiomorph was detected in the *T. europaeum* isolates examined here, with  
6 the exception of the IHEM 25139 strain.

7  
8 Notes — The morphology of *T. europaeum* most closely resembles those of *T. benhamiae* var.  
9 *benhamiae*, *T. japonicum* and *T. mentagrophytes*. *Trichophyton europaeum* shares many morphological  
10 characteristics with *T. japonicum*, including the red/brown pigmentation of the colony reverse colour  
11 on MEA in some strains, the production of conidiophores branched in a pyramidal pattern and abundant  
12 sporulation. The ratio of MAT1-1-1 and MAT1-2-1 strains in the *T. europaeum* strains examined here  
13 was 1:39; by contrast, all *T. japonicum* strains exhibited only the MAT1-1-1 idiomorph (Figures 3, 7).  
14 These two species can be reliably differentiated only by means of molecular methods (ITS and *gapdh*  
15 gene sequences, microsatellite markers, MALDI-TOF MS). *T. benhamiae* var. *benhamiae* differs from  
16 *T. europaeum* and *T. japonicum* in its host spectrum, higher growth rates, especially on MEA and PDA  
17 at 25 °C (Figure 11) and macroconidia characteristics. The differentiation of *T. mentagrophytes* from  
18 *T. europaeum* and *T. japonicum* is sometimes difficult by morphological methods. In general, the  
19 obverse of *T. mentagrophytes* colonies is more intensively coloured in shades of yellow-brown to  
20 brown, and the colony reverse colour is usually dark brown. *T. mentagrophytes* isolates usually produce  
21 abundant spiral hyphae, which are rather rare in *T. europaeum* and *T. japonicum* after 2 weeks. To  
22 differentiate *T. europaeum* from other species, see the descriptions of *T. benhamiae* var. *benhamiae* and  
23 *T. benhamiae* var. *luteum*.

24  
25 ***Trichophyton japonicum*** Cmokova & Hubka, *sp. nov.* — MycoBank XXXX; Figure 20

26  
27 *Etymology.* Refers to the origin of the majority of the examined strains.

28  
29 *Vegetative hyphae* smooth, septate, hyaline, 1.5–4 µm diam (mean ± sd: 2.5 ± 0.6). *Conidiophores*  
30 usually poorly differentiated from hyphae and represented by conidiogenous hyphae with sparse to  
31 numerous short lateral branches; conidiophores branched in a pyramidal (grape-like) pattern relatively  
32 rare. *Microconidia* abundant, born terminally on hyphae, pyriform to clavate, 2.5–5 (3.2 ± 0.4) × 1.5–  
33 3.6 (2.3 ± 0.3) µm. *Macroconidia* rare to abundant, born terminally on hyphae, sparse to abundant  
34 depending on the isolate, consisting of 3–8(–12) cells (median = 5), 11–79 (55.2 ± 12.4) × 5–11 (6.8 ±  
35 1.5) µm, elongated, cigar-shaped, clavate, with a tapering rounded apex and truncate end, macroconidia  
36 consisting of irregular and bloated cells common, long macroconidia easily disintegrate into cylindrical



1 fragments. *Chlamydozoospores* present. *Spiral hyphae* absent to sparse in 14-d-old colonies. *Sexual morph*  
2 unknown.

3

4 *Culture characteristics* — (Colonies in 7 d at 25 °C) Colonies on SAB 16–36 mm diam ( $\varnothing = 23$  mm),  
5 White (#F2F3F4) to Pale Yellowish Pink (#ECD5C5), velvety to floccose, flat with slightly elevated and  
6 furrowed center, edge entire to diffuse, reverse Light Orange (#FAB57F) to Vivid Orange Yellow  
7 (#F6A600) in the marginal part, in some strains Deep Orange Yellow (#C98500) center. Colonies on  
8 MEA 18–30 mm diam ( $\varnothing = 26$  mm), White (#F2F3F4), Light Yellow (#F8DE7E) to Pale Yellowish  
9 Pink (#ECD5C5), floccose to granular, flat, sometimes with an umbonate center, frequently with  
10 concentric ring pattern, margin entire to diffuse, reverse Deep Orange (#BE6516), Strong Reddish  
11 Brown (#882D17) to Vivid Red (#BE0032). Colonies on PDA 16–27 mm diam ( $\varnothing = 23$  mm), White  
12 (#F2F3F4) to Pale Yellowish Pink (#ECD5C5), floccose to granular, occasionally with cottony sectors,  
13 flat or umbonate, margin entire, reverse Deep Orange (#BE6516), Strong Reddish Brown (#882D17)  
14 to Vivid Red (#BE0032). Colonies at 30 °C in 7 d: SAB 32–45 mm diam ( $\varnothing = 38$  mm); MEA 28–37  
15 mm diam ( $\varnothing = 33$  mm); PDA 26–35 mm diam ( $\varnothing = 30$  mm). Colonies at 37 °C in 7 d: SAB 21–38 mm  
16 diam ( $\varnothing = 26$  mm); MEA 32–37 mm diam ( $\varnothing = 35$  mm); PDA 30–35 mm diam ( $\varnothing = 33$  mm).

17

18 *Specimens examined*. SPAIN, human, 1963, P. Miguens (PRM 944416, holotype, dried culture; PRM  
19 944417, isotype; culture ex-type IHEM 17701 = ATCC 28063 = CBS 807.72 = CECT 2894 = RV  
20 14988). BELGIUM, dog, 1971, De Vroey (IHEM 4030 = ATCC 28067 = CBS 809.72 = RV 28105).  
21 JAPAN, rabbit, 2009 (NUBS 09011). JAPAN, Saitama, human, 2000 (VUT 00003-2). JAPAN, Saitama,  
22 rabbit, 1999 (VUT 00002). JAPAN, Saitama, rabbit, 2000 (VUT 00003). JAPAN, human, 2013  
23 (NUBS12001). JAPAN, Hyogo, rabbit, 1997 (VUT 97010). JAPAN, unknown source (JPN3). JAPAN,  
24 unknown source, unknown (JPN6). JAPAN, human, unknown (NUBS13002). CZECHIA, human, tinea  
25 corporis, 2013, N. Mallátová (DMF 35). CZECHIA, human, tinea corporis, 2011, S. Dobiášová (DMF 3061).  
26 CZECHIA, human, tinea corporis, 2012, S. Dobiášová (DMF 2446); *ibid.*, DMF 3031. CZECHIA, human,  
27 tinea corporis, 2013, S. Dobiášová (DMF 1658). CZECHIA, guinea pig (*Cavia porcellus*), 2014, J.  
28 Koubková (KOUB 63). CZECHIA, Pardubice, human, tinea corporis, 2011, K. Mencl (ME 961).  
29 CZECHIA, Prague, human, tinea corporis, 2012, P. Lysková (PL 1773).

30

31 *Distribution and ecology* — *Trichophyton japonicum* is a zoophilic species occurring mostly in rabbits  
32 and guinea pigs. The species is widely distributed in Japan (mostly in rabbits) (Takeda et al. 2012,  
33 Kimura et al. 2015). In Europe it occurs mostly in guinea pigs and less frequently in rabbits and other  
34 hosts. In guinea pigs it is less common than *T. benhamiae* var. *luteum* and *T. europaeum* (see  
35 discussion). It has also been detected in Thailand (Vu et al. 2019), South Korea (P.-L. Sun, pers. comm.)  
36 and Iran (GenBank JX413540; unpublished record).

1 The European and Japan strains of *T. japonicum* (n = 19) examined here were predominantly  
2 obtained from humans (~63 % from females and ~37 % from males, median age 15 years) who mostly  
3 reported contact with rabbits, guinea pigs and dogs. The remaining strains were recovered from the  
4 mentioned animals (Table S1). The infections mostly manifested as tinea corporis (trunk skin 38 %,  
5 extremities 63 %). Only the MAT1-1-1 idiomorph was detected in the *T. japonicum* isolates examined  
6 here.

7  
8 Notes — For the differentiation of *T. japonicum* from similar species, see the description of *T.*  
9 *europaeum*. Only the MAT1-1-1 idiomorph was detected in all examined strains, by contrast all *T.*  
10 *europaeum* strains exhibited the MAT1-2-1 idiomorph except for strain IHEM 25139.

### 11 ***Trichophyton erinacei* clade**

12 ***Trichophyton erinacei*** (J.M.B. Sm. & Marples) Quaipe, J. Clin. Pathol. 19: 178. 1966 — Figure 21

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14  
15  
16 *Vegetative hyphae* smooth, septate, hyaline, 1–3 µm diam (mean ± sd: 1.9 ± 0.8). *Conidiophores*  
17 usually poorly differentiated from vegetative hyphae, conidiophores branched in a pyramidal (grape-  
18 like) pattern present only in some strains, conidia sessile on hyphae or short lateral and terminal  
19 branches. *Microconidia* abundant, mostly clavate or pyriform, 2.9–6.5 (4.3 ± 2.79) × 1.5–3.5 (2.7 ±  
20 0.28) µm diam. *Macroconidia* rare to abundant, predominantly consisting of only two or few cells  
21 (intermediate forms between micro- and macroconidia), max. 5-celled (median = 2), clavate, cigar-  
22 shaped, 6–35 (11 ± 4.52) × 2.5–4.5 (3.4 ± 0.17) µm; intercalary conidia sparse to abundant, cylindrical,  
23 barrel-shaped or irregular. *Chlamydospores* present. *Spiral hyphae* not observed. *Sexual morph*  
24 unknown.

25  
26 *Culture characteristics* — (Colonies in 7 d at 25 °C) Colonies on SAB 19–32 mm diam (∅ = 29 mm),  
27 White (#F2F3F4) to Light Orange Yellow (#F3E5AB) in the centre, flat, finely to coarsely granular,  
28 edge diffuse, reverse Light Orange Yellow (#F3E5AB) to Vivid Yellow (#FADA5E), Deep Reddish  
29 Brown (#882D17) in the centre. Colonies on MEA 19–30 mm diam (∅ = 25 mm), White (#F2F3F4),  
30 flat, finely to coarsely granular, edge diffuse, reverse Light Orange Yellow (#F3E5AB) to Vivid Reddish  
31 Orange (#F38400). Colonies on PDA 11–32 mm diam (∅ = 24 mm) White (#F2F3F4) to Pale Orange  
32 Yellow (#F3E5AB), flat to slightly raised in the center, finely to coarsely granular (velvety to cottony in  
33 some strains), edge diffuse (irregular or submerged in some strains), Light Orange Yellow (#F3E5AB)  
34 to Vivid Yellow (#F3C300), frequently Deep Reddish Brown (#882D17) in the centre. Colonies at 30  
35 °C in 7 d: SAB 25–35 mm diam (∅ = 31 mm); MEA 39–48 mm diam (∅ = 39 mm); PDA 35–42 mm  
36 diam (∅ = 36 mm). Colonies at 37 °C in 7 d: SAB 25–40 mm diam (∅ = 34 mm); MEA 30–37 mm diam  
37 (∅ = 32 mm); PDA 32–34 mm diam (∅ = 33 mm).

1  
2 *Specimens examined.* NEW ZEALAND, hedgehog (*Erinaceus europaeus*), M.J. Marples (ex-holotype  
3 culture CBS 511.73 = ATCC 28443 = IMI 101051 = NCPF 375). THE NETHERLANDS, Delft (Diagnostic  
4 Center SSDZ), arm skin, human, 1979 (CBS 344.79). UNITED KINGDOM, Bristol (General Hospital  
5 Bristol), human, 1972 (IHEM 19619 = RV 28925); *ibid.*, culture IHEM 19621 = RV 28927.

6  
7 *Distribution and ecology* — *Trichophyton erinacei* is a zoophilic species that is common in  
8 wild-living and pet hedgehogs worldwide. The pathogen was originally described in the European  
9 hedgehog (*Erinaceus europaeus*), occurring naturally in the UK and Northern and Western Europe; it  
10 has also been imported to New Zealand and Japan (Smith and Marples 1964, Morris and  
11 English 1969, Takahashi et al. 2003). The African wild-living four-toed hedgehog (*Atelerix*  
12 *albiventris*) is another host of *T. erinacei*. The prevalence of the pathogen is high in both wild-living  
13 and pet hedgehogs, resulting in a significant increase in human infections due to *T. erinacei*,  
14 especially those contracted from pet hedgehogs, in recent years (Abarca et al. 2017, Hubka et al.  
15 2018c, Kargl et al. 2018). The presentation in hedgehog range from asymptomatic infection (Fig. 22)  
16 to extensive involvement of the body surface. The infection is predominantly located on the head and  
17 usually spread slowly (Morris and English 1973, Takahashi et al. 2002, Schauder et al. 2007). In  
18 human, extremities are affected in cca 70–80% of reported cases (Fig. 22), although tinea corporis,  
19 barbae, faciei (Fig. 22), capitis and onychomycosis have been also reported (English et al. 1962,  
20 Piérard-Franchimont et al. 2008, Concha et al. 2012).

21  
22 *Notes* — The morphology of *T. erinacei* resembles that of *T. africanum* and *T. mentagrophytes*.  
23 Compared to *T. mentagrophytes*, with a dark colony reverse colour, the colony reverse colour of *T.*  
24 *erinacei* is pale. These species also differ in the production of spiral hyphae, which are absent in *T.*  
25 *erinacei*, and by the general shape of microconidia, which are mostly globose or subglobose in *T.*  
26 *mentagrophytes*. The species is strongly associated with hedgehogs, and identification is thus only  
27 difficult when isolated from infected humans with incomplete anamnestic data. The closely related taxa  
28 *T. eriotrephon* and *T. verrucosum* are easily distinguishable from *T. erinacei* by their slower growth  
29 rates (Figure 11) and relatively poor sporulation (sporulation usually absent in *T. verrucosum*).  
30 Additionally, *T. eriotrephon* can be differentiated from *T. erinacei* by the production of an intense  
31 reddish-brown pigment and microconidia with variable shapes. Only the MAT1-1-2 idiomorph was  
32 detected among the *T. erinacei* isolates examined here.

33  
34 ***Trichophyton eriotrephon*** Papegaay, Ned. Tijdschr. Geneesk. 69: 885. 1925 — Figure 23

1 *Vegetative hyphae* smooth, septate, hyaline, 1.4–3.2 µm diam (mean ± sd: 2.2 ± 1.0). Well-differentiated  
2 *conidiophores* rare, usually only poorly differentiated from vegetative hyphae, lateral branches arise in  
3 a right-angle to the fertile hyphae, fertile hyphae frequently disintegrate into propagules (intercalary  
4 conidia and microconidia). *Microconidia* abundant, sessile, formed terminally or laterally on fertile  
5 hyphae, or on lateral branches, occasionally in short chains, variable in shape, mostly ovoid or pyriform,  
6 occasionally barrel-shaped, limoniform or irregular, 3.3–6.6 (4.6 ± 0.85) × 2.1–3.7 (3.4 ± 0.41) µm  
7 diam; intercalary conidia common, occasionally arranged in chains, barrel-shaped or irregular.  
8 *Macroconidia* absent. *Spiral hyphae* absent. *Chlamydospores* common. *Sexual morph* unknown.

9

10 *Culture characteristics* — (Colonies in 7 d at 25 °C) Colonies on SAB 17–25 mm diam (∅ = 21 mm),  
11 White (#F5F5F0) or Light Yellowish Brown (#E3D6A1), flat with radially wrinkled centre, velvety to  
12 delicately granular, edge entire, reverse Deep Reddish Brown (#882D17), diffuse pigment Strong  
13 Reddish Brown (#6E2615) produced into the medium (less intense in IHEM 24340). Colonies on MEA  
14 17–25 mm diam (∅ = 21 mm), White (#F5F5F0) to Pale Yellow (#C2B280) in the centre, flat with or  
15 without radially wrinkled centre, velvety to delicately granular, edge submerged and filliform, reverse  
16 Vivid Red (#841B2D) to Deep Reddish Brown (#882D17) (yellow reverse in IHEM 24340). Colonies  
17 on PDA 17–24 mm diam (∅ = 22 mm), White (#F5F5F0) to Light Yellowish Brown (#E3D6A1) in the  
18 centre, flat or umbonate, with raised centre (radially wrinkled in CBS 220.25), velvety or downy, edge  
19 submerged to filliform, reverse Vivid Orange (#F38400) to Strong Yellowish Brown (#80461B) in the  
20 centre. Colonies at 30 °C in 7 d: SAB 22–32 mm diam (∅ = 27 mm); MEA 28–31 mm diam (∅ = 31  
21 mm); PDA 22–25 mm diam (∅ = 24 mm). Colonies at 37 °C in 7 d: SAB 0–3 mm diam (∅ = 1 mm); no  
22 growth on MEA and PDA.

23

24 *Specimens examined*. THE NEDERLANDS, human dermatophytosis, 1925, J. Papegaay (ex-type culture  
25 CBS 220.25). BELGIUM, Marke, dog skin and hair (Jack Russell terrier), 2010 (IHEM 24340).

26

27 *Distribution and ecology* — Insufficient data are available regarding the distribution of *T. eriotrephon*,  
28 which is known from four cases of dermatophytosis in humans (tinea corporis, Netherlands; tinea  
29 manuum and tinea faciei, Iran; tinea barbae, France) (Papegaay 1925, Rezaei-Matehkolaei et al. 2013,  
30 Sabou et al. 2018) and a dog (isolate IHEM 24340 from Belgium). ). It is assumed that *T. eriotrephon*  
31 is a zoophilic species based on its phylogenetic relationships with other zoophilic species and the clinical  
32 manifestations of known infections in humans.

33

34 *Notes* — The morphology of *T. eriotrephon* only slightly resembles species from the *T. benhamiae*  
35 clade in its red-brown colony reverse colour. The conidiophores of *T. eriotrephon* are mostly loose and  
36 poorly branched compared to those of zoophilic species from the *T. benhamiae* clade, with grape-like  
37 conidiophores. Other typical characteristics include the production of a diffuse red-brown pigment on

1 SAB, microconidia with variable shapes and the absence of macroconidia. These characteristics,  
2 together with the absence of or restricted growth at 37 °C, differentiate *T. eriotrephon* from all other  
3 species of the *T. benhamiae* complex. The MAT1-1-1 idiomorph of the mating type gene was detected  
4 in both *T. eriotrephon* isolates examined here.

5

6 ***Trichophyton verrucosum*** E. Bodin, Les champignons parasites de l'homme: 121. 1902 — Figure 24

7

8 *Vegetative hyphae* smooth, septate, frequently inflated, hyaline, 1–2.5 µm diam (mean ± sd: 1.7 ±  
9 1.16). *Conidiophores* rare, poorly differentiated from vegetative hyphae, unbranched or sparsely  
10 branched, conidia sessile on hyphae or born on short lateral branches. *Microconidia* absent or rare,  
11 clavate, 3–6 (4.5 ± 0.7) × 1.9–3.5 (2.9 ± 0.45) µm. *Macroconidia* absent or rare, smooth-walled, clavate  
12 or fusiform with rounded apex and truncate end, usually consisting of 1–4 cells (median = 2), 16–50 ×  
13 4–8 µm. *Chlamydospores* abundant and frequently in the form of chains. *Spiral hyphae* absent. *Sexual*  
14 *morph* unknown.

15

16 *Culture characteristics* — (Colonies in 7 d at 25 °C) Colonies on SAB 18–22 mm diam (∅ = 20 mm),  
17 White (#F5F5F0) to Pale Orange Yellow (#FFF587) or Light Orange Yellow (#FAD6A5), flat, raised  
18 and furrowed, or cerebriform, velvety to slightly powdered, edge entire, lobate, or submerse, reverse  
19 Light Orange Yellow (#F8DE7E) to Deep Orange Yellow (#C9AE5D), dark brown in some strains.  
20 Colonies on MEA 5–18 mm diam (∅ = 13 mm), White (#F5F5F0) to Pale Orange Yellow (#F3E5AB),  
21 raised in the centre, frequently wrinkled, velvety or waxy, edge entire, lobate, or submerse, reverse  
22 Light Orange Yellow (#F8DE7E) to Vivid Orange Yellow (#F6A600), dark brown in some strains.  
23 Colonies on PDA 8–18 mm diam (∅ = 14 mm), White (#F5F5F0) to Pale Orange Yellow (#F3E5AB),  
24 flat or with raised centre, velvety or waxy, edge entire, lobate, or submerse, reverse Light Orange  
25 Yellow (#F8DE7E), dark brown in some strains. Colonies at 30 °C in 7 d: SAB 10–23 mm diam (∅ =  
26 18 mm); MEA 8–10 mm diam (∅ = 9 mm); PDA 9–10 mm diam (∅ = 9 mm). Colonies at 37 °C in 7 d:  
27 SAB 9–15 mm diam (∅ = 10 mm); MEA 11–12 mm diam (∅ = 11 mm); PDA 12–14 mm diam (∅ = 13  
28 mm).

29

30 *Specimens examined*. CZECHIA, Pardubice, dermatophytosis in 21-year-old woman (contact with  
31 cattle), 2011, K. Mencl (CCF 4612). CZECHIA, Hlinsko, dermatophytosis in 58-year-old woman  
32 (contact with cattle), 2011, K. Mencl (CCF 4613). CZECHIA, Tábor, dermatophytosis in 38-year-old  
33 woman (contact with cattle), 2014, N. Mallátová (CCF 4889).

34

35 *Distribution and ecology* — *Trichophyton verrucosum* is a zoophilic species typically found in cattle  
36 and other ruminants (Fig. 22), but it can easily spread to humans and animals, including horses, donkeys,

1 camels, rabbits, dogs, cats, pigs, and even birds (Georg 1960, Dvořák et al. 1965, Ali-Shtayeh et al.  
2 1988, Khosravi and Mahmoudi 2003, Chermette et al. 2008). The species is distributed worldwide, but  
3 the incidence of infections in cattle and man has been decreased in many regions by specific preventive  
4 measures, especially by vaccination programmes or changes in agricultural systems, such as reduction  
5 of the number of cattle in breeding units, and infections in humans have decreased proportionally  
6 (Seebacher et al. 2008, Lund et al. 2014). Human patients usually develop aggressive inflammatory  
7 skin lesions usually located on extremities and head (Fig. 22), which may be accompanied by  
8 constitutional symptoms, such as fever and lymphadenopathy (Silver et al. 2008, Courtellemont et al.  
9 2017). *Tinea barbae* and *capitis* are relatively common clinical forms which can result in irreversible  
10 scarring and alopecia.

11

12 Notes — The morphology of *T. verrucosum* resembles *T. bullosum* and *T. concentricum*. For  
13 distinguishig characters see *T. bullosum* description. Only MAT1-2-1 idiomorph was detected in all  
14 strains examined here and in all strains analyzed by other reserchers (Kano et al. 2014, Kosanke et al.  
15 2018).

16

### 17 ***Trichophyton bullosum* clade**

18

19 ***Trichophyton africanum*** Cmokova & Hubka, *sp. nov.* — MycoBank **XXXX**; Figure 25

20

21 *Etymology.* Refers to the origin of the ex-type strain.

22

23 *Vegetative hyphae* smooth, septate, hyaline, 1–4 µm diam (mean ± sd: 2.2 ± 0.5). *Conidiophores* poorly  
24 differentiated from vegetative hyphae, unbranched or sparsely branched, conidia sessile on lateral or  
25 terminal branches. *Microconidia* abundant, pyriform to clavate, 2.5–5 (4 ± 0.5) × 1.9–2.9 (2.4 ± 0.3)  
26 µm. *Macroconidia* abundant, cigar-shaped, 14–80.5 (64.2 ± 14.4) × 6–11 (8.2 ± 1.2) µm, consisting of  
27 3–9(–13) cells (median = 6). *Chlamydospores* present. *Spiral hyphae* rare or absent. *Sexual morph*  
28 unknown.

29

30 *Culture characteristics* — (*Colonies in 7 d at 25 °C*). Colonies on SAB 30–32 mm diam (∅ = 31 mm),  
31 White (#F2F3F4) to Pale Yellow Green (#F2F3E5), granular, slightly raised in the center, margin  
32 diffuse, reverse Light Orange Yellow (#FBC97F) in the marginal part, Strong Orange (#ED872D) in  
33 the center. Colonies on MEA 28–35 mm diam (∅ = 30 mm), White (#F2F3F4), granular, flat, margin  
34 entire, reverse Light Orange Yellow (#FBC97F). Colonies on PDA 27–28 mm diam (∅ = 28 mm), White  
35 (#F2F3F4) to Pale Yellow Green (#F2F3E5), granular to floccose, slightly raised in the center, margin  
36 entire, reverse Pale Yellow (#F3E5AB) to Pale Orange Yellow (#FAD6A5) in the marginal part, Dark  
37 Orange Yellow (#BE8A3D) in the center. Colonies at 30 °C in 7 d: SAB 40–45 mm diam (∅ = 43 mm);

1 MEA 35–45 mm diam ( $\varnothing = 39$  mm); PDA 35–40 mm diam ( $\varnothing = 36$  mm). Colonies at 37 °C in 7 d: SAB  
2 21–24 mm diam ( $\varnothing = 24$  mm); MEA 20–29 mm diam ( $\varnothing = 23$  mm); PDA 20–22 mm diam ( $\varnothing = 21$  mm).

3  
4 *Specimens examined.* MOZAMBIQUE, human, 1969, M.J. Campos-Magalhaes (PRM 944418, holotype,  
5 dried culture; culture ex-type IHEM 4032 = ATCC 28064 = RV 25293 = CM 3440). BELGIUM, Bruges,  
6 human fingernail, 1978 (IHEM 19628 = RV 40614). SOUTH AFRICA, human skin, 1971, K. Scott  
7 (IHEM 4033 = ATCC 28065 = CBS 808.72 = CECT 2895 = NCPF 456 = RV 27926).

8  
9 *Distribution and ecology* — All three currently known strains are of human origin, but the low number  
10 of isolates does not allow us to draw conclusions about their ecology. The species probably occurs  
11 mainly in Africa.

12  
13 *Notes* — Some aspects of the morphology of *T. africanum* resemble those of zoophilic species from the  
14 *T. benhamiae* clade, *T. erinacei* and *T. mentagrophytes* sensu de Hoog et al. (2017). *Trichophyton*  
15 *africanum* shows a faint apricot colony reverse colour, differing from the intense yellow or red/brown  
16 pigments typical of *T. benhamiae* clade species and *T. mentagrophytes*. The conidiophores of *T.*  
17 *africanum* are unbranched or sparsely branched; when branched, the resulting conidiophores have  
18 usually only few and relatively long lateral branches and are less compact than those of *T. benhamiae*  
19 *var. luteum*, *T. europaeum* and *T. japonicum* (pyramidal/grape-like with many short lateral branches).  
20 *Trichophyton africanum* has conidia of similar lengths to those of *T. benhamiae var. benhamiae* and in  
21 average longer than those of the remaining species from the *T. benhamiae* clade. The differentiation of  
22 this species from *T. erinacei* on the basis of morphology may be difficult, but *T. erinacei* is very strongly  
23 associated with hedgehogs. The most closely related species, *T. bullosum*, can be easily distinguished  
24 by its very slow growth, poor or absent sporulation, and abundant production of chlamydospores. The  
25 ratio of MAT1-1-1 and MAT1-2-1 strains in *T. africanum* was 2:1.

26  
27 ***Trichophyton bullosum*** Lebasque, Les Champignons des Teignes du Cheval et des Bovidés: 53. 1933  
28 — Figure 26

29  
30 *Vegetative hyphae* smooth, septate, inflated, often branched and with knob-like terminations, hyaline  
31 1.5–4  $\mu\text{m}$  diam (mean  $\pm$  sd; 2.7  $\pm$  0.7). *Chlamydospores* abundant, spherical, oval or irregular,  
32 occasionally in chains, 4–9(–20)  $\mu\text{m}$  in diam. *Microconidia* and *macroconidia* not observed in the  
33 isolates examined in this study, but they were observed by Lebasque (1933) under specific conditions.  
34 *Spiral hyphae* absent.

35  
36 *Culture characteristics* — (Colonies in 7 d at 25 °C) Colonies on SAB 11–12 mm diam ( $\varnothing = 12$  mm),  
37 White (#F2F3F4) to Pale Yellowish Pink (#ECD5C5) or Pale Orange Yellow (#FAD6A5), umbonate,

1 radially furrowed, membranous or slightly velvety, edge submerged or filiform, reverse Light Yellow  
2 (#F8DE7E). Colonies on MEA 8–12 mm diam ( $\varnothing = 10$  mm), White (#F2F3F4), Pale Yellow  
3 (#F3E5AB) or Vivid Orange Yellow (#F6A600), flat with raised and cerebriform center, membranous,  
4 edge entire or submerged with dendritic growth, reverse Light Yellow (#F8DE7E). Colonies on PDA  
5 7–9 mm v diam ( $\varnothing = 8$  mm), White (#F2F3F4), Pale Yellowish Pink (#ECD5C5) or Pale Orange Yellow  
6 (#FAD6A5), circular, flat, umbonate, membranous, edge entire, reverse Light Yellow (#F8DE7E).  
7 Colonies at 30 °C in 7 d: SAB 11–14 mm diam ( $\varnothing = 13$  mm); MEA 11–12 mm diam ( $\varnothing = 11$  mm); PDA  
8 12–13 mm diam ( $\varnothing = 12$  mm). Colonies at 37 °C in 7 d: SAB 8–10 mm diam ( $\varnothing = 9$  mm); MEA 9–10  
9 mm diam ( $\varnothing = 10$  mm); PDA 9–10 mm diam ( $\varnothing = 9$  mm).

10  
11 *Specimens examined.* FRANCE, horse, J. Lebasque (ex-type culture, CBS 363.35 = LP 770). CZECHIA,  
12 skin lesions in horse, 2013, P. Lysková (CCF 4831). EGYPT, near Cairo, skin lesion in donkey (*Equus*  
13 *asinus*), 2015, A. Peano (CCF 5730).

14  
15 *Distribution and ecology* — *Trichophyton bullosum* is a zoophilic species known from infections in  
16 donkeys and horses (Fig. 27). It is distributed in Europe, North Africa and the Middle East (Lebasque  
17 1933, Sitterle et al. 2012, Lysková et al. 2015, Sabou et al. 2018).

18  
19 *Notes* — Due to its slow grow rate, *T. bullosum* strongly resembles *T. verrucosum* and *T. concentricum*.  
20 These species either do not sporulate or sporulate poorly (especially on sugar-rich media such as SAB)  
21 but produce abundant chlamydospores, frequently in the form of chains. All mentioned species are  
22 relatively strongly associated with their hosts and/or with a typical clinical manifestation (cattle  
23 ringworm caused by *T. verrucosum*; dermatophytosis caused by *T. bullosum* in horses and donkeys;  
24 tinea imbricata caused by *T. concentricum* in humans). Therefore, detailed anamnestic data can facilitate  
25 their identification. Molecular genetic methods may be necessary to verify the identification of some  
26 isolates. For the differentiation of *T. bullosum* from the most closely related species, *T. africanum*, see  
27 the description of *T. africanum*. Only the MAT1-1-1 idiomorph was detected among the *T. bullosum*  
28 isolates examined here.

## 30 **DISCUSSION**

### 31 **Species delimitation issues in *Trichophyton***

32 Species delimitation in dermatophytes is based on a polyphasic approach (Gräser et al. 2008) combining  
33 ecological (distribution, host range) and clinical data, the analysis of DNA sequence data, the macro-  
34 and micromorphological examination of cultures, physiological and biochemical tests and mating tests.  
35 However, the application of the individual components of this concept is limited in many species  
36 complexes due to specific problems. As a result, the “polyphasic” approach is commonly applied in a  
37 restricted form in practice.



1 Phenotypic criteria are usually relatively effective in routine diagnostics for major  
2 dermatophyte species or species complexes. However, as in other fungi, we have found similarities  
3 between species or morphotypes across unrelated dermatophytes, resulting in misdiagnosis in practice  
4 (Summerbell 2011, Lysková et al. 2015, Uhrlaß et al. 2018). There is also considerable intraspecific  
5 phenotypic variability in other species or species complexes that is not correlated with molecular  
6 taxonomy (Heidemann et al. 2010, Su et al. 2019, Kandemir et al. 2020). Moreover, the success rate of  
7 phenotypic identification frequently depends on the age of isolates because of the rapid degeneration of  
8 important portions of cultures (de Hoog et al. 2017). Consequently, it can be difficult to maintain and  
9 reproduce phenotypic characters over decades for the purposes of taxonomic studies.

10 The high level of clonality in many primary pathogenic dermatophytes with a presumed recent  
11 origin is also associated with an extremely low level of genetic intraspecific variability. Consequently,  
12 there is a lack of sufficiently variable DNA sequence markers for the differentiation of some species  
13 and, therefore, ambiguities in the definition of their boundaries (de Hoog et al. 2017). Phenomena such  
14 as incomplete lineage sorting or occasional hybridization and introgression may further complicate the  
15 species delimitation of evolutionarily recently diverged species with semi-permeable reproductive  
16 barriers (Taylor et al. 2015, Steenkamp et al. 2018, Matute and Sepúlveda 2019). The divergence  
17 between these young species may be hidden when using some classical protein-coding phylogenetic  
18 markers. Neutrally evolving or noncoding DNA regions, such as microsatellites, introns and intergenic  
19 spacers, which accumulate mutations more rapidly, were shown to reveal the evolutionary trajectories  
20 of primary pathogenic dermatophytes with higher success (Gräser et al. 2008, Mochizuki et al. 2017,  
21 Hubka et al. 2018c).

22 The specific problems in species delimitation in *Trichophyton* can be demonstrated by the  
23 example of the *T. mentagrophytes* and *Trichophyton rubrum* complexes. It was generally assumed that  
24 the differentiation of zoophilic *T. equinum* (main host = horse) from closely related anthropophilic *T.*  
25 *tonsurans* would be possible based on the ecological preferences, nutritional requirements, and MAT  
26 gene idiomorphs of the fungi (Woodgyer 2004, Summerbell et al. 2007). Kandemir et al. (2020)  
27 examined 67 isolates and found that none of the five selected phylogenetic markers were able to  
28 unambiguously separate these species (probably due to incomplete lineage sorting) according to  
29 differences in their MAT genes, ecology and nicotinic acid requirements. It is postulated that these  
30 species evolved very recently and that the speciation process might not yet be complete (Kandemir et  
31 al. 2020). Another taxonomically problematic species pair is *T. mentagrophytes/T. interdigitale*.  
32 According to the traditional concept promoted by de Hoog et al. (2017), *T. mentagrophytes* is a  
33 zoophilic species in which both MAT idiomorphs are present in the population, resulting in relatively  
34 high intraspecific genetic variability. By contrast, anthropophilic *T. interdigitale* is a clonal lineage  
35 (consisting only of the MAT1-1-1 idiomorph) that is almost exclusively associated with onychomycosis  
36 and tinea pedis. Although the correlation between the genotype and the clinical manifestation or source  
37 of isolates has been repeatedly demonstrated, the correlation between ITS genotype and phenotype is

1 relatively poor (Heidemann et al. 2010, Pchelin et al. 2016, Dhib et al. 2017). Currently, the molecular  
2 diagnosis of these species is mostly based on several unique sites in the ITS region, and phylogenies  
3 usually resolve *T. mentagrophytes* as para- or polyphyletic with *T. interdigitale* (Heidemann et al. 2010,  
4 Nenoff et al. 2019, Pchelin et al. 2019, Singh et al. 2019, Taghipour et al. 2019, Hainsworth et al. 2020).  
5 Both species names remain in use, due to the epidemiological consequences associated with different  
6 sources of infections in particular. The laboratory diagnosis of *T. mentagrophytes* and *T. interdigitale*  
7 and that of *T. equinum* and *T. tonsurans* are further complicated by inaccurate or even impossible  
8 species differentiation using MALDI-TOF MS (Nenoff et al. 2013, da Cunha et al. 2018, Dukik et al.  
9 2018, Suh et al. 2018, Hedayati et al. 2019).

10 Very similar species delimitation issues complicate the taxonomy of the anthropophilic *T.*  
11 *rubrum* complex, encompassing clonal lineages showing differences in their distribution and the clinical  
12 manifestation of associated infections (Gräser et al. 2000, de Hoog et al. 2017). The majority of  
13 molecular studies relying on the variability in the ITS region and microsatellite markers have revealed  
14 some support for 2–4 lineages (i.e., *T. rubrum*, *T. violaceum* and/or *T. soudanense* and/or *T. yaoundei*),  
15 but the number of species and their boundaries are still under debate (Gräser et al. 2007, Su et al. 2019,  
16 Packeu et al. 2020). MALDI-TOF MS showed promising results in the differentiation of these  
17 species/lineages (Packeu et al. 2020).

18 Detailed genomic, epigenetic and multigene phylogenetic studies on a large number of samples  
19 can resolve delimitation issues between these species in the future (Zhan et al. 2018, Pchelin et al. 2019,  
20 Singh et al. 2019). SNP detection by whole-genome sequence typing can be used to infer the genetic  
21 relatedness of *Trichophyton* isolates. This approach will ultimately become one of the methods of  
22 choice in the future with decreasing costs (Hadrich and Ranque 2015). Currently, the sequencing of ITS  
23 rDNA and population genetic markers such as microsatellites (Kaszubiak et al. 2004, Gräser et al. 2007,  
24 Pasquetti et al. 2013) or mixed-marker approaches (Abdel-Rahman et al. 2010) offers higher  
25 discriminatory power in the species differentiation of primary pathogenic dermatophytes compared to  
26 MLST approaches based on the currently available loci.

27

### 28 **Disentangling the taxonomy of the *T. benhamiae* complex based on a polyphasic approach**

29 In this study, we encountered similar problems to those mentioned in the previous chapter in the *T.*  
30 *benhamiae* complex. However, a polyphasic approach combining independent molecular genetic  
31 markers (four DNA loci and 10 microsatellite loci) with phenotypic features and ecological data helped  
32 to overcome the majority of obstacles to species delimitation.

33 We showed that isolates that were designated in the past as the European-American race of *T.*  
34 *benhamiae* harbour five taxa (three species and two varieties). The strains with the so-called white  
35 phenotype do not represent monophyletic entities and correspond to *T. benhamiae* var. *benhamiae*, *T.*  
36 *japonicum* and *T. europaeum*, while the yellow phenotype strains correspond to *T. benhamiae* var.  
37 *luteum*. Isolates of the African race of *T. benhamiae* referred to as *T. africanum* herein are

1 phylogenetically distant and are most closely related to *T. bullosum*. The main characteristics of these  
2 species and features that are useful for their identification are schematically summarized in Figure 28.

3         None of the four **sequence markers** alone was able to unequivocally differentiate all species  
4 within the *T. benhamiae* complex and provide accurate identification in 100% of cases. The ITS region  
5 contained a diagnostic position for all nine species, but the differentiation of *T. europaeum* and *T.*  
6 *japonicum* relied on a single substitution. In addition, the identification of isolate IHEM 25139, with a  
7 probable hybrid origin, failed as described above. The *gapdh* gene was useful for differentiation  
8 between *T. europaeum* and *T. japonicum*, but some pairs of sister species shared identical sequences  
9 (i.e., *T. benhamiae* and *T. concentricum*, *T. verrucosum* and *T. eriotrephon*). The *tefl-α* gene  
10 differentiated all species except for *T. europaeum* and *T. japonicum*. The *tubb* gene presented the least  
11 discriminatory power and failed to differentiate species within the *T. benhamiae* clade but could be used  
12 for species identification in the *T. erinacei* and *T. bullosum* clades. Insufficient discriminatory power of  
13 the *tubb* gene has been reported in many other *Trichophyton* species (Suh et al. 2018, Kandemir et al.  
14 2020, Packeu et al. 2020). The unique substitutions observed within the DNA loci of the *T. benhamiae*  
15 clade species will be the basis for reliable species identification in practice. The taxonomic significance  
16 of these unique sites is unambiguous, as they correspond to independent microsatellite markers and  
17 phenotypic and ecological data, indicating the reproductive isolation of recognized taxa.

18         While sequence markers were shown to be useful for the diagnosis of *T. benhamiae* complex  
19 species, they were not able to distinguish the two varieties of *T. benhamiae*. The only intraspecific  
20 variation was a single substitution in the *tefl-α* gene. This substitution was able to differentiate all *T.*  
21 *benhamiae* var. *luteum* isolates from the majority of *T. benhamiae* var. *benhamiae* strains, with the  
22 exception of two isolates from cluster C2, probably due to incomplete lineage sorting between these  
23 recently diverged lineages. The results of other analyses clearly indicated that *T. benhamiae* var. *luteum*  
24 is an emerging entity that is distinct both qualitatively (at the population genetic level and according to  
25 phenotypic differences) and ecologically (showing different hosts and distributions). The differentiation  
26 of this taxon has clinical relevance, due to which we decided to reassign it as variety of the nearest  
27 recombining ancestor, *T. benhamiae* var. *benhamiae*. We chose this conservative approach rather than  
28 the proposals of a new species because of the impossibility of distinguishing this entity using available  
29 DNA sequence markers, as is the current standard in fungal taxonomy.

30         In contrast to DNA sequence data, **population genetic analysis** based on the newly developed  
31 microsatellite typing scheme clearly separated all species in the *T. benhamiae* clade (Figures 5–6),  
32 including *T. benhamiae* var. *benhamiae* and *T. benhamiae* var. *luteum*. Similarly, pilot **MALDI-TOF**  
33 **MS** analysis was able to identify specific peaks for all species and varieties in the *T. benhamiae* clade,  
34 suggesting that this increasingly popular method can be used for species identification in clinical  
35 practice, but the analysis of additional isolates will be needed to generate a more robust database and  
36 confirm our preliminary observations.

1           **Phenotypic and ecological data** added another important piece to the taxonomic puzzle.  
2 *Trichophyton benhamiae* var. *luteum* can be identified by its slow growth on all media at all  
3 temperatures and its uniform phenotype (yellow reverse side of colonies and absence of macroconidia;  
4 all strains exhibit only mating type MAT1-1-1). The closely related *T. benhamiae* var. *benhamiae* is  
5 only found in the USA (mostly dogs) and exhibits strikingly different colonies with a brown to red-  
6 brown reverse side, macroconidium production and larger microconidia than *T. benhamiae* var. *luteum*.  
7 This variety shows the most rapid growth among the species from the *T. benhamiae* clade; isolates with  
8 both MAT gene idiomorphs were detected among the examined strains. *Trichophyton europaeum* is the  
9 second most common species from the *T. benhamiae* complex occurring in Europe and is responsible  
10 for human and guinea pig infections. While *T. japonicum* is currently responsible for the majority of  
11 human and animal (rabbits and guinea pigs) infections in Japan, it also occurs in Europe at low  
12 frequencies. Reliable differentiation of these species is only possible by molecular methods (Figure X).  
13 *Trichophyton japonicum* and *T. europaeum* differ strikingly in the distribution of mating type genes in  
14 their populations. Detailed distinguishing characteristics of particular species are listed in the  
15 Taxonomy section, and some important characteristics are summarized in Figure 28.

16

### 17 **Speciation through host switching and the extinction of opposite mating type partners**

18 The assessment of species boundaries via **mating experiments** (revealing biological compatibility)  
19 played an important role in the delimitation of many early species and the discovery of their sexual  
20 states. This approach based in principle on the biological species concept (BSC) is generally highly  
21 applicable in geophilic dermatophytes (Dawson and Gentles 1962, Stockdale 1964, Padhye and  
22 Carmichael 1972, Choi et al. 2012, Hubka et al. 2015a). By contrast, the results of biological  
23 compatibility assessment can considerably disagree with the concept of classical species of  
24 anthropophilic and zoophilic dermatophytes. These species are evolutionarily young, and their  
25 phylogenetic divergence preceded the development of reproductive barriers, as demonstrated by  
26 interspecific hybrid induction between various primary pathogenic *Trichophyton* species in vitro  
27 (Kawasaki et al. 2009, Anzawa et al. 2010, Kawasaki et al. 2010, Kawasaki 2011). However, it is highly  
28 unlikely that this kind of hybridization occurs naturally due to the different ecological niches of species,  
29 and the results of in vitro mating assays therefore cannot be extrapolated to a natural scenario.  
30 Additionally, the **ratio of mating-type gene idiomorphs** is usually extremely imbalanced or one  
31 idiomorph is missing in the majority of anthro- and zoophilic dermatophytes (Metin and Heitman  
32 2017, Kosanke et al. 2018). This fact further limits or even prevents the possibility of using BSCs in  
33 the delimitation of these species. A similar phenomenon was observed by our group in all species from  
34 the *T. benhamiae* complex (Figure 28), suggesting that the loss of opposite mating-type partners was  
35 an important driver of their evolution. The ancestors of many currently recognized pathogenic  
36 dermatophytes were likely sexually reproducing geophilic species and zoophilic species on free-living  
37 mammals (sexually reproducing, e.g., in soil surrounding burrows) with balanced ratios of opposite

1 mating type individuals (Gräser et al. 2006, Summerbell 2011). Adaptation to a new host is probably a  
2 unique event in the evolution of many anthropo- and zoophilic dermatophytes, resulting in the  
3 extinction of one mating partner in the whole population of these species. Only some “clonal” offshoots  
4 of ancestral sexual dermatophytes probably maintain ongoing populations and follow independent  
5 evolutionary trajectories towards speciation (Gräser et al. 2006). Alternatively, the extinction of one  
6 MAT gene in a population of dermatophytes may be caused by the preferential spread of strain(s)  
7 exhibiting an advantageous combination of alleles associated with higher virulence/transmission  
8 potential. Such a successful genotype may be significantly dominant in conditions with almost exclusive  
9 asexual transmission and may displace other genotypes. Such a situation is very likely to lead to an  
10 imbalance in the MAT gene ratio or even the loss of one MAT gene in the population. The extinction  
11 of strains belonging to one mating type is, for instance, observed in some populations of *M. canis*  
12 (Sharma et al. 2007), and different levels of virulence linked with mating-type idiomorphs have been  
13 repeatedly documented in fungal pathogens (Yue et al. 1999, Chang et al. 2000, Cheema and Christians  
14 2011).

15 In the *T. benhamiae* clade, clonal reproduction is the dominant mode of dissemination (Dg, H,  
16 DW indices), and recombination is rare or absent in almost all populations according to the I<sub>A</sub>. Despite  
17 the fact that only MAT1-2-1 idiomorph strains were present within *T. europaeum* strains, the null  
18 hypothesis of random mating was not rejected (Table S6, Figure 9), suggesting the existence of recent  
19 recombination events in this species. As *T. japonicum* and *T. europaeum* consist of a single mating type  
20 and no recent recombination or gene flow has occurred between them, they should be conceptualized  
21 as separate, albeit clonal species, despite potential in vitro interbreeding (Summerbell 2002, Gräser et  
22 al. 2006). The disruption of gene flow between *T. benhamiae* clade species was demonstrated by  
23 fixation indices (F<sub>ST</sub> or G<sub>ST</sub>) (Table S5, Table S6).

24 In the *T. benhamiae* complex, there are at least two **possible sexual ancestors** of “clonal”  
25 species: *T. benhamiae* var. *benhamiae* and *T. africanum*, based on the presence of both MAT gene  
26 idiomorphs. While the ecology of *T. africanum* is poorly known, reservoirs of *T. benhamiae* var.  
27 *benhamiae* exist in free-living animals. It has been detected in the North American porcupine  
28 (Takahashi et al. 2008, Needle et al. 2019), but its host spectrum can be broader and may include  
29 members of family Canidae, as evidenced by repeated isolation from dogs (Ajello and Cheng 1967,  
30 Sieklucki et al. 2014) and patients who have come into contact with foxes (Tan et al. 2020). Due to  
31 close phylogenetic proximity, *Trichophyton benhamiae* var. *benhamiae* was very likely a common  
32 ancestor of at least some taxa in the *T. benhamiae* clade, especially anthropophilic *T. concentricum*  
33 (only MAT1-1-1) and zoophilic *T. benhamiae* var. *luteum* (only MAT1-1-1). The low genetic diversity  
34 within *T. benhamiae* var. *luteum* together with its recent origin (according to the DW index) may  
35 indicate a founder effect in the recent past. This may suggest that the origin of *T. benhamiae* var. *luteum*  
36 lies in North America and that one or a few strains were recently introduced to Europe.

1           The only exception among the examined isolates was strain IHEM 25139 (= RV 14387 = ATCC  
2 28061 = CBS 806.72 = IFM 54422), isolated in 1963 by M. Takashio from guinea pig in France. This  
3 strain, identified here as *T. europaeum* based on the *gapdh* gene, shared some microsatellite alleles with  
4 *T. japonicum*. It also presented the MAT1-1-1 idiomorph of the MAT gene, typical of *T. japonicum* or  
5 *T. benhamiae* var. *benhamiae* cluster C3, and an atypical ITS1 region sequence with six substitutions  
6 compared to other *T. europaeum* strains, some of which are at positions crucial for the differentiation  
7 of *T. benhamiae* clade species (Figure S2). It is possible that this strain originated from **hybridization**  
8 between *T. europaeum* and *T. japonicum*. The ecological niches of these species partially overlap, as  
9 they both occur in guinea pigs and some other animals that are frequently maintained together. In  
10 addition, the coinfection of guinea pigs with two species or morphotypes has been repeatedly  
11 documented (Kupsch et al. 2017, Bartosch et al. 2019). In such cases, the exchange of genetic  
12 information may likely occur not only through hybridization during saprophytic growth outside the host  
13 (possibly followed by introgressive hybridization) but also during coinfection of the same host through  
14 a parasexual cycle (anastomosis of hyphae, mitotic crossing-over and haploidization). Another strain  
15 with an ITS sequence identical to IHEM 25139 is IHEM 19622 (= RV 14389), which was not examined  
16 by our group (GenBank MK298816). These two strains with identical provenance were noted by  
17 Takashio to be atypical compared to other examined *A. benhamiae* isolates because of the less compact  
18 texture of their colonies (Takashio 1974). These strains represent unique material for studying natural  
19 hybridization in dermatophytes. Their origin and genomic arrangement remain to be elucidated by  
20 genomic studies. The absence of these genotypes among the more recently isolated strains examined  
21 here and by others (no additional occurrence in GenBank) suggests that they were short-lived and were  
22 replaced by more successful genotypes.

23

#### 24 **Geographical distribution of *T. benhamiae* clade species**

25 To understand the **global distribution of the newly reassigned species in the *T. benhamiae* clade**, we  
26 analysed several hundred ITS rDNA sequences deposited in GenBank. The analysis enabled the  
27 identification of these records to the species level based on the species-specific substitutions in the ITS  
28 region. This fact further supported the feasibility of the novel taxonomic classification proposed here.  
29 The ecological data resulting from the analysis were used as a basis for mapping the distribution of *T.*  
30 *benhamiae* clade species (Figure 29; Table S10). The main limitations are the unavailability of  
31 epidemiological and DNA data from America, many Asian countries and Africa. As a result, the  
32 majority of analysed ITS sequences are from European countries and Japan, where dermatophyte  
33 research has a long tradition, and DNA-based identification is more commonly used. Additionally, it is  
34 not possible to distinguish two varieties of *T. benhamiae* based on the ITS region, but macro- and  
35 micromorphological characters described in some studies enable clear distinction of the varieties; the  
36 variety characteristics described below refer to such cases.

1 In Europe, guinea pigs are hosts of all three pathogens, among which *T. benhamiae* var. *luteum*  
2 is the most prevalent, followed by *T. europaeum* and *T. japonicum*. The ITS-based identification of 30  
3 *T. benhamiae* strains from guinea pigs from a single veterinary institution in Prague (Czech Republic)  
4 between 2014–2019 revealed a 24:4:2 ratio of these pathogens (Hubka and Prausová, unpubl. data).  
5 The corresponding ratio of these pathogens in human Czech patients is very similar, ~27:5:1 (Hubka et  
6 al. 2014, Hubka et al. 2018b, Hubka et al. unpubl. data). In addition to guinea pigs, another important  
7 reservoir of *T. japonicum* are rabbits, while other animal hosts of *T. benhamiae* clade members seem to  
8 be much less important.

9 Based on current knowledge, it is clear that white-phenotype strains of *T. benhamiae* occurred  
10 in Europe before epidemic spread of *T. benhamiae* var. *luteum*. The oldest European white-phenotype  
11 strains representing *T. japonicum* are IHEM 4030 (collected before 1988 in Belgium) and IHEM 17701  
12 (collected before 1997 in Spain). The oldest white-phenotype strains representing *T. europaeum* were  
13 collected before 1988 in Finland (Aho 1980) (Table S10) and more recently from Switzerland, in 2002  
14 (IHEM 20159, IHEM 20161, IHEM 20162, IHEM 20163). The identity of other old white-phenotype  
15 strains reported in various European countries from the 1960s to 2000 (Figure 1) is unclear due to the  
16 unavailability of isolates and/or sequence data. Both *T. japonicum* and *T. europaeum* were subsequently  
17 detected in Japan and some other countries (Figure 29, Table S10). Outbreaks of infections caused by  
18 *T. benhamiae* var. *luteum* now seem to be limited to Europe, but an increasing number of infections can  
19 be expected in non-European countries due to its recent introduction to other continents (Hiruma et al.  
20 2015, de Freitas et al. 2019).

21 Zoophilic *T. benhamiae* clade members have probably been brought into Japan with imported  
22 animals on several occasions and spread in Japan by the transportation of animals by breeders or pet  
23 shops, as suggested in a series of publications (Kano et al. 1998, Mochizuki et al. 2001, Takeda et al.  
24 2012, Hiruma et al. 2015). The most prevalent species in Japan and South Korea (Jun et al. 2004, Lee  
25 et al. 2018, and pers. comm. with PL Sun) is *T. japonicum*. Other species are probably much less  
26 common: *T. europaeum* has been detected in guinea pig (unknown year of isolation) (Takeda et al.  
27 2012), *T. benhamiae* var. *benhamiae* was imported to a Japanese zoo from Canada and the USA (in  
28 2000 and 2002) with North American Porcupines (Takahashi et al. 2008), and *T. benhamiae* var. *luteum*  
29 was detected in 2012 in common degu (Hiruma et al. 2015).

30 In addition to Europe and Japan, *T. benhamiae* var. *luteum* was recently reported in Brazil (de  
31 Freitas et al. 2019, Santana et al. 2020). *Trichophyton benhamiae* var. *benhamiae* was confirmed only  
32 in North America in our study but was also recently reported in China (Tan et al. 2020).

33 Animal trade certainly plays an important role in the spread of zoonotic dermatophytes to new  
34 geographic areas. It also erases original geographic areas of a species distribution. Consequently, it is  
35 difficult to trace the origin of particular species. The current worldwide distribution and prevalence of  
36 infections caused by *T. benhamiae* clade members are poorly known due to insufficient overall  
37 surveillance of dermatophytosis supported by molecular-based identification. This problem pertains to

1 both human and veterinary medicine. In addition, our knowledge of the ecology of these pathogens is  
2 mostly limited to domestic animals and pets, and little is known about potential wild-living hosts.  
3 Therefore, any hypothesis about the species origin is based on very incomplete data and needs to be  
4 refined by future research.

#### 6 **Genotyping and surveillance of emerging pathogens in the *T. benhamiae* complex**

7 The emergence and rapid spread of *T. benhamiae* in Europe in the last decade and the recent detection  
8 of this species in many other countries has been one of the major public health events in the field of  
9 zoonotic superficial mycoses in recent years. This fact underscores the need for the One Health  
10 integrative approach and closer collaboration between the veterinary profession, dermatologists,  
11 epidemiologists and public health personnel (Nenoff et al. 2014, Hubka et al. 2018c). Infected and  
12 frequently asymptomatic animals may act as a recurrent source of infections in other animals and  
13 humans. Interdisciplinary cooperation is needed to establish effective preventive measures for the  
14 control of infections.

15 Genotyping techniques are often employed to gain insight into the dynamics of disease  
16 transmission, determine the source and routes of infections, confirm or rule out outbreaks, recognize  
17 virulent strains and regional and global changes in genotype patterns and evaluate the effectiveness of  
18 control measures (Ranjbar et al. 2014). Other common issues in dermatophytes concern the  
19 differentiation of relapse versus reinfection and the determination of whether the infection is caused by  
20 one or more strains and if genotypes differ in their clinical manifestation. Many methods have been  
21 developed for the subtyping of dermatophytes, but a significant number of them are now obsolete, and  
22 their utility is frequently limited due to poor reproducibility or unsatisfactory strain differentiation  
23 (Abdel-Rahman 2008, Mochizuki et al. 2017, Hubka et al. 2018c). MLST typing approaches have been  
24 widely applied to many fungal pathogens (Meyer et al. 2009, Debourgogne et al. 2012, Bernhardt et al.  
25 2013, Maitte et al. 2013, Prakash et al. 2016), but no such typing scheme has been evaluated and  
26 developed for dermatophytes, and the currently available loci usually lack sufficient discriminatory  
27 power to study the population structure of *Trichophyton* and *Microsporum* species in detail.  
28 Microsatellite markers are still among the most effective tools available for the subtyping of  
29 dermatophytes. Typing schemes have been developed for a limited number of species, including only  
30 *T. rubrum* (Gräser et al. 2007), *Nannizzia persicolor* (Sharma et al. 2008) and *M. canis* (Sharma et al.  
31 2007, Pasquetti et al. 2013).

32 Polymorphisms in *T. benhamiae* (American-European race) were previously investigated by  
33 the RFLP analysis of the NTS region, which produced 11 different patterns in 46 isolates; this method  
34 successfully confirmed laboratory-acquired infections as well as familial outbreaks transmitted from  
35 pets (Mochizuki et al. 2002, Takeda et al. 2012). In this study, we developed a microsatellite typing  
36 scheme consisting of ten variable markers. This new typing scheme is currently the most powerful tool  
37 for the subtyping of *T. benhamiae* clade species. It is easy to use and cost-effective due to its multiplex



1 design. It is possible that the modified scheme can be used in other species in the *T. benhamiae* complex.  
2 Our preliminary data showed that at least 6 of 10 markers (CT21b, TAG16, TC20, TCA16, TC19,  
3 TC17a) are useful for the subtyping of another emerging pathogen, *T. erinacei*.

4 The establishment of global databases based on largely comparable data, such as that from  
5 microsatellites, SNPs and DNA sequences, is desirable. Such databases would enable us to understand  
6 the global epidemiology of dermatophytes and monitor changes in genotype spectra on a global scale.  
7 Although high-throughput sequencing facilities are now widely available and increasingly used even in  
8 the epidemiology of fungal infections, this option has not yet been exploited in dermatophytes.

9 The prevalence and spread of emerging pathogens from the *T. benhamiae* complex require close  
10 monitoring, particularly because infection rates in the principal hosts (guinea pigs, hedgehogs, and  
11 others) are high. The new taxonomic classification and microsatellite typing scheme proposed in this  
12 study will enable the monitoring of changes in the frequencies of individual species and genotypes. It  
13 will help to evaluate the results of preventive measures and interventions and is a basic prerequisite for  
14 the preparation of epidemiological studies.

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1 **FIGURE LEGENDS**

2 **Figure 1.** Chronology of reports of *Trichophyton benhamiae* phenotypes from various countries.  
3 Yellow-phenotype isolates correspond to *T. benhamiae* var. *luteum* proposed in this study. White-  
4 phenotype strains correspond to *T. benhamiae* var. *benhamiae* and two novel species proposed here: *T.*  
5 *europaeum* and *T. japonicum*. The reports are mostly sorted according to the phenotypic characters of  
6 cultures reported by the authors and, in more recent studies, by a combination of DNA sequencing and  
7 morphology. The icons of the hosts are explained in Figure S1.

8  
9 **Figure 2.** Multilocus phylogeny of the *Trichophyton benhamiae* complex inferred with the maximum  
10 likelihood method based on the *gapdh*, *tubb*, ITS rDNA and *tef1- $\alpha$*  loci (alignment characteristics,  
11 partitioning scheme and substitution models are listed in Tab S3). Maximum likelihood bootstrap values  
12 and Bayesian posterior probabilities are appended to the nodes; only support values higher than 70%  
13 and 0.90, respectively, are shown. The ex-type strains are designated with a superscripted T.  
14 *Trichophyton rubrum* CBS 202.88 was used as the outgroup.

15  
16 **Figure 3.** Haplotype network of the *Trichophyton benhamiae* clade based on multilocus data  
17 (*gapdh*, *tubb*, ITS rDNA and *tef1- $\alpha$*  loci). Haplotypes are indicated by circles whose sizes correspond  
18 to the number of analysed strains, and dashes on the connecting lines indicate substitutions (indels are  
19 excluded). The upper figure shows the species identity and genotypic diversity, the middle figure shows  
20 the distribution of MAT gene idiomorphs, and the lower figure shows the geographic distribution of  
21 particular genotypes.

22  
23 **Figure 4.** Plot of mean genotypic diversity as a function of the number of microsatellite loci.

24  
25 **Figure 5.** The population structure of the *Trichophyton benhamiae* clade (ten microsatellite loci, 318  
26 isolates). The neighbour-joining tree was calculated from the multilocus microsatellite profiles using  
27 the Jaccard distance matrix measure in FAMD 1.3 (Schlueter Harris 2006) and is used solely for the  
28 comprehensive presentation of the results. Genetic structure was revealed with STRUCTURE software  
29 by Bayesian clustering (the peak of  $\Delta K$  was observed at  $K = 6$ ); clones were discarded from the analysis;  
30 the number of isolates representing each haplotype is indicated in parentheses following the isolate  
31 number; the geographic origin of the isolates representing particular haplotypes is indicated using  
32 abbreviations: Europe (Eu), Japan (Jpn), United States of America (USA). Individual haplotypes are  
33 represented by horizontal bars; the colours were attributed according to the clusters delimited by  
34 STRUCTURE.

35

1 **Figure 6.** Population structure of the *Trichophyton benhamiae* clade revealed by the analysis of ten  
2 microsatellite loci in 318 strains. The NeighborNet network was built with FAMD 1.3 software and  
3 visualized in SplitsTree 4.13 using the Jaccard index-based distance matrix (Delta score: 0.1778, Q-  
4 residual score: 0.01222). The assignment of strains to main clusters and species is indicated by different  
5 colours. The labels of each cluster show the geographic origin of strains with the number of isolates and  
6 main host(s). The icons of the hosts are explained in Figure S1.

7

8 **Figure 7.** Phylogenetic tree of the *Trichophyton benhamiae* clade revealed by the analysis of ten  
9 microsatellite loci in 318 strains constructed in FAMD software using a Jaccard index-based distance  
10 matrix. Coloured circles display the genotype diversity of the ITS, *gapdh* and *tef1- $\alpha$*  loci and the  
11 distribution of MAT gene idiomorphs (blue: MAT1-1-1; pink: MAT1-2-1) across *Trichophyton*  
12 *benhamiae* clade species. Isolate numbers are displayed in Figure S6.

13

14 **Figure 8.** Histograms showing the frequency of pairwise genetic differences within the population of  
15 *Trichophyton benhamiae* var. *benhamiae* clusters C2 and C3 (A); *Trichophyton benhamiae* var. *luteum*  
16 (B); *Trichophyton japonicum* (C); *Trichophyton europaeum* (D).

17 **Figure 9.** Histogram of the simulated index of association ( $I_A$ ) from 10 000 permutations of  
18 randomization tests under a null model of allelic recombination; the observed values of  $I_A$  are indicated  
19 with an arrow.

20 **Figure 10.** Overview of the macromorphology of the *Trichophyton benhamiae* complex taxa on three  
21 media (SAB, MEA and PDA) cultivated for 14 days at 25 °C.

22

23 **Figure 11.** Growth rates of *Trichophyton benhamiae* complex members on three media (SAB, MEA  
24 and PDA) and at three different temperatures (25, 30 and 37 °C, on SAB only) after 7 days of  
25 cultivation; circles represent median values and the whiskers span the minimum and maximum values.

26

27 **Figure 12.** Length and width of microconidia in taxa belonging to the *Trichophyton benhamiae*  
28 complex. The horizontal lines indicate mean value and interquartile range, whiskers span the 5% and  
29 95% percentiles and circles extreme outliers.

30

31 **Figure 13.** Principal component analysis (PCA) of morphological characteristics. The two major axes  
32 of the plot show all variables, including the growth rates (cultivation on MEA, SAB, and PDA at 25,  
33 30 and 37 °C) and microconidium sizes (mean values of length and width) (A). The correlation matrix  
34 shows the Pearson correlation coefficients between variables such as growth rates (three different media  
35 and temperatures) and microconidia sizes (length and width). A darker colour indicates stronger



1 correlations, which means that all variables within a growth rate or microconidium size group were  
2 strongly correlated (B), indicating the possibility of reducing the number of variables.

3

4 **Figure 14.** MALDI-TOF mass spectra in the *Trichophyton benhamiae* clade members; only variable  
5 regions are shown. Comparison of spectra in the species of the former Americano-European race (*T.*  
6 *benhamiae* var. *benhamiae* and *T. benhamiae* var. *luteum*, *T. europaeum* and *T. japonicum*) and the  
7 African race (*T. africanum*) (A). Comparison of *T. europaeum*, *T. japonicum* and *T. concentricum* (B).  
8 Comparison of spectra of *T. concentricum* and two varieties of *T. benhamiae* (C-D).

9

10 **Figure 15.** Macromorphology and micromorphology of *Trichophyton benhamiae* var. *benhamiae*.  
11 Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract  
12 agar (C, D) and Potato dextrose agar (E, F). Conidiophores bearing microconidia (G-I) and  
13 macroconidia (J); macroconidia (K-P), frequently with mycelial fragments at one or both ends (K-N,  
14 P); microconidia (R); spiral hyphae (S). Scale bars = 20 µm.

15

16 **Figure 16.** Macromorphology and micromorphology of *Trichophyton benhamiae* var. *luteum*.  
17 Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract  
18 agar (C, D) and Potato dextrose agar (E, F). Conidiophores bearing microconidia (G-L); microconidia  
19 (M). Scale bars = 20 µm.

20

21 **Figure 17.** Clinical presentation of infections caused by *Trichophyton benhamiae* clade species in  
22 guinea pigs and humans. Guinea pigs: area of alopecia with scaling located in the temporal area (A)  
23 and on the back of guinea pig (B); areas with scaling on the ear (C); itchy area of alopecia behind the  
24 ear (D); weeping lesion under the eye (E). Zoonotic infections in humans: tinea corporis located on  
25 the thigh (F) and chest (G), tinea faciei (H), tinea barbae (I), tinea capitis profunda (J, K).

26

27 **Figure 18.** Macromorphology and micromorphology of *Trichophyton concentricum*. Colonies after  
28 three weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract agar (C, D) and  
29 Potato dextrose agar (E, F). Vegetative hyphae (G-L), frequently consisting of inflated cells and  
30 containing intercalar or terminal chlamydospores (H, I), occasionally proliferating in a zigzag pattern  
31 (K, L). Scale bars = 20 µm.

32

33 **Figure 19.** Macromorphology and micromorphology of *Trichophyton europaeum*. Colonies after two  
34 weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract agar (C, D) and  
35 Potato dextrose agar (E, F). Conidiophores bearing microconidia (G-I); macroconidia (J-M);  
36 microconidia (N); spiral hyphae (O, P). Scale bars = 20 µm.

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**Figure 20.** Macromorphology and micromorphology of *Trichophyton japonicum*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract agar (C, D) and Potato dextrose agar (E, F). Conidiophores bearing microconidia (G-K); macroconidia (L-P); microconidia (R); spiral hyphae (S, T). Scale bars = 20 µm.

**Figure 21.** Macromorphology and micromorphology of *Trichophyton erinacei*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract agar (C, D) and Potato dextrose agar (E, F). Conidiophores bearing microconidia (G-J) and macroconidia (intermediate forms) (K); macroconidia (L-N); free microconidia and macroconidia (two-celled intermediate forms) (N); intercalary conidia – marked with arrows (P). Scale bars = 20 µm.

**Figure 22.** Clinical presentation of infections caused by *Trichophyton erinacei* clade species in animals and humans. *Trichophyton erinacei*: four-toed hedgehog (*Atelerix albiventris*) without apparent clinical signs of infection (A), a source of tinea corporis infection in a pet breeder (Lysková et al. 2018); tinea faciei (B) and tinea corporis on the left forearm (C). *Trichophyton verrucosum*: discrete, scaling patches of hair loss located on the head and neck of cattle (D, E) and goat (F); tinea corporis on the forearm (G), infection that affected scalp skin after previous injury - the situation after surgical removal of necrotic parts (H).

**Figure 23.** Macromorphology and micromorphology of *Trichophyton eriotrephon*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract agar (C, D) and Potato dextrose agar (E, F). Conidiophores bearing microconidia and intercalary conidia (G-K); microconidia and intercalary conidia with variable shape (L). Scale bars = 20 µm.

**Figure 24.** Macromorphology and micromorphology of *Trichophyton verrucosum*. Colonies after three weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract agar (C, D) and Potato dextrose agar (E, F). Clumps of vegetative hyphae (G); chlamydospores in chains (H, I); macroconidia (J-M); conidiophores (fertile hyphae) with sessile microconidia (N, O); microconidia (P). Scale bars = 20 µm.

**Figure 25.** Macromorphology and micromorphology of *Trichophyton africanum*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract agar (C, D) and Potato dextrose agar (E, F). Conidiophores bearing microconidia (G-K); macroconidia (L-N); microconidia (O). Scale bars = 20 µm.

1 **Figure 26.** Macromorphology and micromorphology of *Trichophyton bullosum*. Colonies after three  
2 weeks of cultivation at 25 °C on Sabouraud's dextrose agar (A, B), Malt extract agar (C, D) and  
3 Potato dextrose agar (E, F). Chladospores in chains and free chlamyospores (G); thick-walled  
4 vegetative hyphae with numerous intercalary or terminal chlamyospores (H, I); detail of colony with  
5 submerged, dendritic growth on Sabouraud's dextrose agar supplemented with cycloheximide and  
6 chloramphenicol after 3 months of cultivation at 25 °C (L); vegetative hyphae with terminal  
7 chlamyospores (M, N). Scale bars = 20 µm.

8

9 **Figure 27.** Clinical presentation of infections caused by *Trichophyton bullosum*: patches of hair loss  
10 in the saddle area, shoulders, hip bones, withers and upper chest of a horse (A), isolate CCF 4831  
11 (Lysková et al. 2015); scaling patches of hair loss located on the head, chest and legs of a donkey (B-  
12 E), isolate CCF 5730.

13

14 **Figure 28.** Overview of selected data on ecology, phenotype and population genetics plotted on the  
15 simplified four-gene phylogeny of the *Trichophyton benhamiae* species complex. The icons of the hosts  
16 are explained in Figure S1.

17

18 **Figure 29.** Geographic distribution of species belonging to the *Trichophyton benhamiae* clade based  
19 on ITS rDNA available in GenBank database (Table S10). The main primary host(s) of species in  
20 different continents are marked by icons (explained in Figure S1).

21

## 22 **LEGENDS TO SUPPLEMENTARY FIGURES**

23 **Figure S1.** Legend for the host icons used in this study.

24

25 **Figure S2.** Maximum likelihood tree based on ITS region sequences. Maximum likelihood bootstrap  
26 values are appended to the nodes; only support values higher than 70% are shown; the ex-type strains  
27 are designated with a superscripted T; *Trichophyton rubrum* CBS 202.88 was used as the outgroup.  
28 Clades with >5 identical sequences are collapsed; positions refer to the alignment available in the Dryad  
29 digital repository.

30

31 **Figure S3.** Maximum likelihood tree based on *gapdh* gene sequences. Maximum likelihood bootstrap  
32 values are appended to the nodes; only support values higher than 70% are shown; the ex-type strains  
33 are designated with a superscripted T; *Trichophyton rubrum* CBS 202.88 was used as the outgroup.  
34 Clades with >5 identical sequences are collapsed; positions refer to the alignment available in the Dryad  
35 digital repository.

36

1 **Figure S4.** Maximum likelihood tree based on *tef1- $\alpha$*  gene sequences. Maximum likelihood bootstrap  
2 values are appended to the nodes; only support values higher than 70% are shown; the ex-type strains  
3 are designated with a superscripted T; *Trichophyton rubrum* CBS 202.88 was used as the outgroup.  
4 Clades with >5 identical sequences are collapsed; positions refer to the alignment available in the Dryad  
5 digital repository.

6  
7 **Figure S5.** Maximum likelihood tree based on *tubb* gene sequences. Maximum likelihood bootstrap  
8 values are appended to the nodes; only support values higher than 70% are shown; the ex-type strains  
9 are designated with a superscripted T; *Trichophyton rubrum* CBS 202.88 was used as the outgroup.  
10 Clades with >5 identical sequences are collapsed.

11  
12 **Figure S6.** Phylogenetic tree of the *Trichophyton benhamiae* clade revealed by the analysis of ten  
13 microsatellite loci in 318 strains constructed in FAMD software using a Jaccard index-based distance  
14 matrix. Coloured circles display the genotype diversity of the ITS, *gapdh* and *tef1- $\alpha$*  loci and the  
15 distribution of MAT gene idiomorphs across *Trichophyton benhamiae* clade species.

16