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ONE HEALTH APPROACH TO UNDERSTAND EMERGING ZOONOTIC PATHOGENS IN THE *TRICHOPHYTON BENHAMIAE* COMPLEX

Doctoral thesis

Supervisor: MUDr. Mgr. Vít Hubka, Ph.D.

Prague 2023

"The emergence and rapid spread of yellow phenotype strains of the fungus T. benhamiae in European guinea pigs and their breeders have been major public health events in the field of zoonotic superficial mycoses" (hotpot.ai)

"We assume that the origin of yellow phenotype epidemic strains is in North America where closely related strains of T. benhamiae have their natural reservoir in free-living animals, such as North American porcupines" (hotpot.ai)

“The presence of two partially isolated populations of fungus *T. erinacei* which may co-evolve with different hosts (European vs African hedgehogs), has been hypothesized.” (picture: hotpot.ai)

PROHLÁŠENÍ

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její významná část nebyla předložena k získání jiného nebo stejného akademického titulu.

DECLARATION

I declare that this thesis has not been submitted for the purpose of obtaining the same or any other academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of included publications and manuscripts. All literature sources I used when writing this thesis have been properly cited.

Adéla Čmoková
Praha, Czech Republic, October 2023

STATEMENT OF CONTRIBUTION

This thesis is the cumulative work consisting of the five published papers in which I am the first author or co-author. Details on my contribution to the each of the papers included in thesis are specified in detail below.

Čmoková A, Kolařík M, Dobiáš R, Hoyer LL, Janouškovcová H, Kano R, Kuklová I, Lysková P, Machová L, Machová L, Maier T, Mallátová N, Man M, Mencl K, Nenoff P, Peano A, Prausová H, Stubbe D, Uhrlaß S, Větrovský T, Wiegand C, and Hubka. 2020. Resolving the taxonomy of emerging zoonotic pathogens in the *Trichophyton benhamiae* complex. Fungal Diversity 104: 333–387.

Contribution of Adéla Čmoková: Conception, data collection, statistical analysis, interpretation of results and draft manuscript preparation.

Čmoková A, Rezaei-Matehkolaei A, Kuklová I, Kolařík M, Shamsizadeh F, Ansari S, Gharaghani M, Miňovská V, Najafzadeh MJ, Nouripour-Sisakht S, Yaguchi T, Zomorodian K, Zarrinfar H, and Hubka V. 2021. Discovery of new *Trichophyton* members, *T. persicum* and *T. spiraliforme* spp. nov., as a cause of highly inflammatory tinea cases in Iran and Czechia. Microbiology Spectrum 9: e00284-00221.

Contribution of Adéla Čmoková: Conception, data collection, statistical analysis, interpretation of results and draft manuscript preparation.

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Contribution of Adéla Čmoková: Data collection, interpretation of results and co-writing the manuscript.

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Contribution of Adéla Čmoková: Conception, data collection, statistical analysis, interpretation of results and draft manuscript preparation.

Shamsizadeh F, Ansari S, Zarei Mahmoudabadi A, Hubka V, **Čmoková A**, Guillot J, Rafiei A, Zomorodian K, Nouripour-Sisakht S, Diba K, Mohammadi T, Zarrinfar H, and Rezaei-Matehkolaei A. 2021. In vitro antifungal susceptibility patterns of Trichophyton benhamiae complex isolates from diverse origin. Mycoses 64: 1378-1386

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Title
One Health approach to understand emerging zoonotic pathogens in the *Trichophyton benhamiae* complex

Title in Czech
Komplexní přístup k pochopení nových zoonotických patogenů z komplexu *Trichophyton benhamiae*

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CONTENTS

ABSTRACT

The *Trichophyton benhamiae* complex encompasses several zoonotic pathogens that are of increasing concern to the scientific community due to their epidemic spread among pets and their owners. Of particular concern is the sudden appearance and rapid spread of *T. benhamiae* yellow phenotype strains in Europe. Considerable genetic and phenotypic variability has been revealed in the pathogens from the complex, yet the species limits and host spectra have not been clearly elucidated. To explore the diversity, epidemiology, and taxonomy of the *T. benhamiae* complex, my colleagues and I formed an international, cross-disciplinary team and applied a holistic One Health approach.

We collected a large dataset of strains from several continents, along with associated data about host, clinical picture, and locality. Due to the high level of clonality in commonly used DNA sequence markers, I developed a new typing schema based on ten microsatellite markers and four DNA sequence markers. We then used a polyphasic approach combining data from independent analyses to decide on species limits in the *T. benhamiae* complex, including phylogenetic and population-genetic analyses, phenotypic and physiological analyses, mating-type gene characterization, ecological data and MALDI-TOF mass spectrometry analysis. Thanks to our approach we were able to introduce four new taxa, including three new species and one new variety (**Paper 1**). The variety rank, *T. benhamiae* var. *luteum*, was proposed for epidemic yellow phenotype strains of *T. benhamiae* that occur in guinea pigs in Europe. We assume that this variety was introduced to Europe from North America where closely genetically related strains of *T. benhamiae* var. *benhamiae* have their natural reservoir. The new variety has a partially overlapping geographic range and hosts with populations of two closely related pathogens which we have described as new species, *T. europaeum* and *T. japonicum* spp. nov. These pathogens were repeatedly documented on guinea pigs in Europe since the 1960s in comparison to the new variety which was first identified in Europe in 2002. The third new species *T. africanum* sp. nov. was proposed for the African race of *T. benhamiae*. In addition, thanks to our extensive data collection, we detected the presence of two additional taxa which we later described as new species (**Paper 2**). The first species, *Trichophyton persicum*, was documented as a cause of human dermatophytosis with an exclusively Iranian distribution, while the second species, *T. spiraliforme,* was a causal agent of human dermatophytosis in Czechia, presumably transmitted from a dog. In contrast, even after extensive data collection from wild hedgehogs of the genus *Erinaceus* (**Paper 3**) and wild and pet hedgehogs of the genus *Atelerix*, we could not confirm the full separation of previously recognized host specific populations in *T. erinacei*. We verified previously reported genetic differences and traced some phenotypic differences between populations associated with *Erinaceus* and *Atelerix* hosts including the size of conidia and different antifungal susceptibility patterns. However, we observed evidence of ongoing gene flow between these populations and presented *T. erinacei* as a single species (**Paper 4**). Finally, we assessed antifungal susceptibility patterns in members of the complex. We did not detect any resistances to commonly used antifungals and we did not find any significant differences in

susceptibility patterns between particular species, except for the two mentioned *T. erinacei* populations (**Paper 5**).

Due to considerable phenotypic similarity and phenomena such as incomplete lineage sorting, occasional hybridization and introgression, we demonstrated the need to follow a polyphasic approach in species delimitation of dermatophytes. Neutrally evolving and non-coding DNA regions showed significantly higher discriminatory power compared to conventional protein-coding loci. The new taxonomic classification and microsatellite typing scheme proposed in this study will enable the monitoring of changes in the frequencies of individual species and genotypes. It will help to evaluate the results of preventive measures and interventions and is a basic prerequisite for the preparation of epidemiological studies. To help implement our knowledge into clinical practice, we prepared a monographic overview of the complex and summarized diagnostic options.

ABSTRAKT

Komplex druhů *Trichophyton benhamiae* zahrnuje několik zoonotických patogenů, které vědeckou komunitu stále více znepokojují kvůli svému epidemickému šíření mezi domácími zvířaty a jejich majiteli. Zvláštní obavy vzbuzuje náhlé objevení a rychlé šíření kmenů se žlutým fenotypem druhu *T. benhamiae* v Evropě. U patogenů z tohoto komplexu byla zjištěna značná genetická a fenotypová variabilita, avšak druhové hranice a hostitelské spektrum nebyly dosud jasně objasněny. Abychom prozkoumali rozmanitost, epidemiologii a taxonomii komplexu *T. benhamiae*, vytvořili jsme s kolegy mezinárodní mezioborový tým a uplatnili holistický "One Health" přístup.

Shromáždili jsme rozsáhlý set kmenů z několika kontinentů spolu se souvisejícími údaji o hostitelích, klinickém obrazu a lokalitě. Vzhledem k vysoké míře klonality u běžně používaných DNA markerů jsem vyvinula nové typizační schéma založené na deseti mikrosatelitních a čtyřech sekvenčních markerech. Pro zjištění druhových hranic v komplexu *T. benhamiae* jsme využili polyfázický přístup, ve kterém jsme zohlednili data z fylogenetických, a populačně-genetických, fenotypových a fyziologických analýz spolu s charakterizací příslušnosti k jedné ze dvou křížících idiomorf a s ekologickými údaji. Díky našemu přístupu se nám podařilo uvnitř komplexu popsat čtyři nové taxony, včetně tří nových druhů a jedné nové variety (**Publikace 1**). Stupeň variety, *T. benhamiae* var. *luteum*, byl navržen pro epidemické kmeny *T. benhamiae* se žlutým fenotypem, které se vyskytují u morčat v Evropě. Předpokládáme, že tato varieta byla do Evropy zavlečena ze Severní Ameriky, kde se vyskytují blízce geneticky příbuzné kmeny *T. benhamiae* var. *benhamiae*. Geografický areál a hostitele této nové variety se částečně překrývají s populacemi dvou blízce příbuzných patogenů, které jsme popsali jako nové druhy, *T. europaeum* a *T. japonicum* spp. nov. Tyto patogeny byly v Evropě opakovaně dokumentovány na morčatech od 60. let 20. století naopak nová varieta byla zde poprvé zdokumentována až v roce 2002. Třetí nový druh, *T. africanum*, byl navržen pro africkou rasu *T. benhamiae*. Kromě toho jsme díky rozsáhlému sběru dat zjistili přítomnost dalších dvou taxonů, které jsme později popsali jako nové druhy (**Publikace 2**). První druh, *T. persicum*, byl zdokumentován jako původce lidské dermatofytózy s rozšířením omezeným na Írán, zatímco druhý druh, *T. spiraliforme*, byl původcem lidské dermatofytózy v Česku, pravděpodobně přenesené od psa. Naopak u *T. erinacei* se ani po rozsáhlém sběru dat z volně žijících ježků rodu *Erinaceus* (**Publikace 3**) a z volně žijících ježků a domácích mazlíčků rodu *Atelerix* nepodařilo potvrdit úplné oddělení dříve rozpoznaných hostitelsky-specifických populací. Ověřili jsme dříve uváděné genetické rozdíly a vysledovali jsme některé fenotypové rozdíly mezi populacemi spojenými s hostiteli rodu *Erinaceus* a *Atelerix*, včetně velikosti konidií a rozdílné citlivosti k antimykotikům. Pozorovali jsme však důkazy o pokračujícím toku genů mezi těmito populacemi a proto jsme *T. erinacei* ponechali jako jediný druh (**Publikace 4**). Nakonec jsme vyhodnotili citlivost druhů z komplexu k antimykotikům. Nezjistili jsme žádnou rezistenci k běžně používaná léčiva a nenašli jsme žádné významné rozdíly v citlivosti mezi jednotlivými druhy, s výjimkou dvou zmíněných populací *T. erinacei* (**Publikace 5**).

Vzhledem ke značné fenotypové podobnosti a výskytu jevů, jako je neúplné třídění linií, příležitostná hybridizace a introgrese, jsme demonstrovali důležitost použití polyfázického přístupu k vymezení druhových hranic. Neutrálně se vyvíjející a nekódující oblasti DNA vykazovaly výrazně vyšší rozlišovací schopnost mezi druhy a izoláty ve srovnání s konvenčními protein-kódujícími lokusy. Nová taxonomická klasifikace a mikrosatelitové typizační schéma navržené v této studii umožní sledovat změny ve frekvencích jednotlivých druhů a genotypů. Také pomohou ve vyhodnocování výsledků preventivních opatření a intervencí a jsou základním předpokladem pro přípravu epidemiologických studií. Abychom pomohli implementovat naše poznatky do klinické praxe, připravili jsme monografický přehled komplexu a shrnuli diagnostické možnosti.

ONE HEALTH APPROACH TO DERMATOPHYTOSIS

Dermatophytes are the most common fungal pathogens worldwide. The prevalence of dermatophytosis - the diseases caused by dermatophytes - can reach dozens of percent in both human and animal populations (Havlickova *et al.* 2008; Cafarchia *et al.* 2010; Ahdy *et al.* 2016; Kupsch *et al.* 2017). Despite strong interest in this group for over 150 years, our understanding of its biology, ecology and evolution is still fragmentary. While infrequent pathogens and geophilic species are often neglected by research, the main knowledge about pathogenic members is restricted to only some areas with a strong dermatophyte research tradition such as European countries, Japan, and Iran. As with other organisms, the basis of any research on a group such as dermatophytes is well-defined study units, e.g., well-resolved taxonomy down to the species or population level. Discussions over classification which should reflect the natural classification as closely as possible are conducted separately for each fungal group due to varying speed of evolution in different organisms. In dermatophytes, a clinically significant group, the determination of species boundaries has been extensively discussed, and opinions on the number of accepted species have changed over the years. The rapid development of molecular methods has revolutionized the taxonomy of dermatophytes allowing for better tracking of gene flow in populations, but it has also made the classification highly unstable (de Hoog et al. 2023). The large number of name changes has made the work of clinicians much more difficult, and to this day, the dialogue between clinicians and scientists remains partly disrupted, representing a significant problem that prevents the use of a unified taxonomy in practice and taxonomic research, thus compromising stability. Scientists often require a system based on natural classification; clinical mycologists, in particular, require a user-friendly system suitable for easy diagnostics and epidemiological monitoring (de Hoog et al. 2023). Scientists often generate a large number of taxonomic novelties without any discussion with the community, while others do not intervene in taxonomy, despite having convincing results, as they do not want to disrupt an already problematic relationship with clinicians. This reluctance of clinical mycologists to accept novelties and of scientists to draw clear taxonomic conclusions has led to the introduction of unnatural, misleading, and unstable classifications. As a result, epidemiological studies, which bring valuable information about the clinical picture, ecology, and distribution of dermatophyte species, are often based on ambiguous identifications making follow-up or comparative research difficult. Consequently, crucial information, such as pathogen-host-environment associations may be partly lost. To resolve this situation, a holistic approach and close cooperation between all involved parties is necessary.

Defining and resolving species boundaries is problematic for clinical fungi such as dermatophytes for many reasons, the most crucial being the clonal nature of some dermatophytes due to the exclusion of the sexual process as a response to adaptation to their pathogenic life cycle (Gräser *et al.* 2006). Like other pathogens, dermatophytes are forced to constantly evolve in response to host-pathogen battles by sprouting new clonal offspring genotypes. These new emerging genotypes cannot

initially be distinguished ecologically or clinically from other genotypes of the same species (Taghipour et al. 2019). Such differentiation, not only phylogenetic distance alone, is crucial for describing a species according to the currently used species concept in dermatophytes - a polyphasic approach (de Hoog *et al.* 2023). This brings us back to the scientist-clinician conflict; these genotypes need to be distinguished for the purpose of epidemiological monitoring but at the same time they do not meet the conditions for describing them as a new species. Introducing a system of complexes to avoid a clear taxonomic conclusion may be one way to temporarily deal with this problem. However, our goal should be a natural classification based on species as the basic units and which follows the flow of genes in populations. Therefore, to define species, we need to complement phylogenetical data with follow up taxonomical and epidemiological research, e.g., further investigation of host spectra, global dissemination, and antifungal resistance of individual members. Such action requires close cooperation between taxonomists, environmental biologists, the veterinary profession, dermatologists, epidemiologists and public health personnel (Nenoff *et al.* 2014; Hubka *et al.* 2018b; Bontems *et al.* 2020). This cooperation should be further deepened over time. Resolved taxonomy brings us back to the practical aspect, i.e., its implementation in clinical practice including health care workers training and designing appropriate identification procedures which is crucial for acquiring additional, accurate epidemiological data. As a result, the increasing number of epidemiological studies substantiated by correct species identification using molecular data and the fast-growing amount of sequence data in global databases, will lay the groundwork for follow up research and effective diagnosis of these diseases (Gräser *et al.* 2018). The collaborative and cross-disciplinary approach which includes all aspects of research and community science research (e.g., taxonomy, population genetics, diagnostics, ecology and antifungal susceptibility testing) is called the One Health approach. This integrative approach is rare in dermatophyte research, even though it is increasingly being promoted within the scientific community nowadays.

In this thesis, the One Health approach was applied to infections caused by emerging pathogens from the Trichophyton benhamiae complex, combining taxonomy, ecology, population genetics, environmental science, veterinary and human medicine, antifungal susceptibility testing, and diagnostics (Papers 1–5).

INTRODUCING THE *TRICHOPHYTON BENHAMIAE* COMPLEX

The members of the complex are mostly animal pathogens, including pets and livestock animals (Hubka *et al.* 2018b). The only clearly anthropophilic species in the complex is *T. concentricum*, a cause of tinea imbricata in rural indigenous populations in the tropics (Bonifaz *et al.* 2004; Pihet *et al.* 2008; Bonifaz & Vazquez-Gonzalez 2011). *Trichophyton verrucosum*, a cause of dermatophytosis in cattle and other ruminants, is one of the best-known members of the complex. It has a worldwide distribution and causes economic losses in the food, hide and skin industries (Chermette *et al.* 2008; Bond 2010). The incidence of infections in cattle has decreased in many regions in response to vaccination programs or changes in agricultural systems, and the rate of infections in humans has decreased proportionally (Seebacher et al. 2008; Lund et al. 2014). Conversely, a lack of prophylaxis accounts for the high infection rates observed in countries such as Italy (Moretti *et al.* 2013). *Trichophyton verrucosum* grows slowly in culture and often produces only chlamydospores as its main microscopic characteristic. In this respect, it is superficially very similar to *T. bullosum*, which causes infections in donkeys and horses (Sitterle *et al.* 2012; Lysková *et al.* 2015; Sabou *et al.* 2018; Peano *et al.* 2022). There is scant data on the host spectrum of *T. eriotrephon*, which is only known from several poorly documented cases of dermatophytosis in humans and dogs (Rezaei-Matehkolaei *et al.* 2013; Sabou *et al.* 2018). The remaining two zoophilic species, *T. benhamiae* and *T. erinacei*, are currently considered emerging pathogens, as their incidence as a cause of infections in pets and humans has increased significantly in the last two decades (Hubka *et al.* 2018b).

Before a thorough taxonomic revision, *Trichophyton benhamiae* (syn. *Arthroderma benhamiae*) was a species with remarkably high intraspecific variability on many levels, including morphology, mating compatibilities and host preferences that differed between continents. Specifically, the "Americano-European" and "African" races are recognized based on mating compatibilities, and "yellow" and "white" phenotype strains of the "European-American race" differ by their prevailing colony colour (Takashio 1974; Kano *et al.* 2008; Symoens *et al.* 2013). Human infections due to *T. benhamiae* manifest as highly inflammatory tinea of glabrous skin, and tinea capitis (Nenoff *et al.* 2014; Skořepová *et al.* 2014). The infections are transmitted to humans mainly from guinea pigs but also from many other minor hosts such as dogs (mostly reported from North America), rabbits, cats, and various rodents (Aho 1980; Fréalle *et al.* 2007; Takeda *et al.* 2012; Sieklucki *et al.* 2014; Hiruma *et al.* 2015; Ziółkowska *et al.* 2015; Needle *et al.* 2019; Peano *et al.* 2022b; Smagulova *et al.* 2023).

Human infections due to *T. erinacei* are mostly transmitted from pet hedgehogs (African pygmy hedgehogs, four-toed hedgehogs, and *Atelerix albiventris*) and less commonly from wild European hedgehogs (genus *Erinaceus*). They usually manifest as tinea manuum or tinea corporis localized on fingers, hands, wrists, or forearms. The incidence of infections began to increase with changes in breeders' preferences who are increasingly buying hedgehogs as pets. However, wild hedgehogs may also present a risk for people, especially in wildlife rehabilitation centres or for dogs that may

occasionally encounter free-living hedgehogs (Le Barzic *et al.* 2021). Considerable genetic and phenotype variability has previously been reported in *T. erinacei*, and the presence of two populations has been hypothesized (Gräser *et al.* 2006; Heidemann *et al.* 2010). The first population colonizing European hedgehogs (*Erinaceus europeus*) is usually described as having only individuals of one mating type, displaying urease-negativity and showing different ITS genotypes in comparison with the potentially sexually-reproducing and urease-positive population reported from African four-toed hedgehogs (*Aterelix albiventris*) (Takashio 1974; Takahashi *et al.* 2003; Schauder *et al.* 2007; Heidemann *et al.* 2010). Such differences suggest the existence of partially isolated populations that may co-evolve with different hosts (Gräser *et al.* 2006).

These facts raised many questions prompting the research included in this thesis. It was not clear if different *T. erinacei* populations had differentiated to the extent that they could meet the criteria for delimitation of separate species. Similarly, we investigated the hypothesis that ecologically and phenotypically distinct populations of *T. benhamiae* form separate taxa. Although there were many indications that *T. benhamiae* and *T. erinacei* are species complexes, there were several problems that had prevented previous scientists from making a definitive taxonomic decision. Among them, poor sampling (lack of strains from different localities) and insufficient knowledge of global population structure and intraspecific variability were among the most important obstacles. Additionally, clonal offspring lineages of some pathogenic species may, if analysed individually, give the impression of separate taxa in phylogenetic analyses. Furthermore, the majority of samples are often collected from human clinical specimens, with only a disproportionately smaller part coming from veterinary samples or natural wildlife populations.

The aim of the study was to collect as large of a set of strains as possible to develop a classification based on the biological characteristics of populations that is understandable to clinicians and effective in clinical practice. Simplification for clinicians should involve the development of clear diagnostic guides and easy-to-use tools for identification and capturing the infraspecific variability for further monitoring purposes.

In total, myself and my colleagues, collected almost 500 molecularly identified strains from the T. benhamiae complex in collaboration with medical and veterinary institutions, scientists, and scientific collections around the world (Paper 1-4). Extensive sampling uncovered the presence of structured populations, including new taxa. Notably, high phenotypic and genetic intraspecific variability was detected in T. benhamiae. In addition to the "yellow" and "white" phenotype strains, considerable genetic variability was found in "white" phenotype strains from different geographical locations (Papers 1 and 2). Moreover, we found significant intraspecific variability in T. erinacei strains obtained from wild-living hedgehogs and pet animals from different geographical locations (Papers 3 and 4).

TRICHOPHYTON BENHAMIAE, AN EMERGING PATHOGEN IN EUROPE

The emergence and rapid spread of *T. benhamiae* in Europe over the last decade as well as its recent detection in many other countries have been major public health events in the field of zoonotic superficial mycoses. Until the end of the millennium, *Trichophyton benhamiae* was considered of little clinical importance. However, after 2010, it became one of the most common agents of zoonotic dermatophytosis in Europe (Symoens *et al.* 2013; Nenoff *et al.* 2014; Uhrlaß *et al.* 2015; Hubka *et al.* 2018a; Sabou *et al.* 2018). A strikingly high incidence of zoonotic infections, mostly due to the "yellow phenotype" strains contracted from guinea pigs, was reported in various European countries (Symoens *et al.* 2013; Hubka *et al.* 2014; Nenoff *et al.* 2014; Uhrlaß *et al.* 2015). The first documented cases of infections due to these strains were recorded between 2002 and 2008 in France and Switzerland (Contet-Audonneau & Leyer 2010; Charlent 2011; Khettar & Contet-Audonneau 2012; Symoens *et al.* 2013). The first cases in Germany and the Czech Republic were described shortly before 2010, and the pathogen quickly became epidemic in the following years. Currently, *T. benhamiae* is the most important agent of dermatophytoses transmitted from animals in the Czech Republic and Germany (Hubka *et al.* 2014; Nenoff *et al.* 2014; Uhrlaß *et al.* 2015; Hubka *et al.* 2018a; Kupsch *et al.* 2020). It has been shown that the prevalence of the pathogen in guinea pig breeds and pet shops can reach up to 90 % (Drouot *et al.* 2009; Kupsch *et al.* 2017; Overgaauw *et al.* 2017; Guillot *et al.* 2018; Bartosch *et al.* 2019). The presence of asymptomatic infections in animal hosts contributes to the successful spread of the pathogen between animals kept in groups, facilitating transmission to pet owners, breeders, and pet shop workers, and leading to familial outbreaks.

In contrast to yellow-phenotype strains, white-phenotype strains had been reported worldwide long before the appearance of yellow phenotype strains. Sporadic human and animal infections had been described from various European countries, Japan, and the USA before the widespread dispersal of yellow phenotype strains in Europe (Ajello & Cheng 1967; Takashio 1974; Aho 1980; Hejtmánek & Hejtmánková 1989; Kano *et al.* 1998).

The origin of the epidemic yellow phenotype strains of *T. benhamiae* and the reason for the sudden increase in the incidence of human and animal infections in Europe remain unknown. As guinea pig breeding has been popular in Europe for decades, this epidemic cannot be explained by a change in pet owner behaviour. Therefore, it has been hypothesized that the spread of a new virulent and highly transmissible genotype/lineage has occurred (Čmoková 2015; Hubka *et al.* 2018b). The occurrence of *T. benhamiae* infections in non-European countries is generally poorly known, aside from individual reported cases. This is mostly due to insufficient surveillance and the lack of long-term epidemiological studies supported by molecular-based identification of dermatophytes (misidentification of *T. benhamiae* with *T. mentagrophytes* is common when only phenotype is used for identification).

By uncovering the population structure of T. benhamiae, we have attempted to answer the question of where the origin of the yellow phenotype strains lies. Based on a literature review and searching in

sequence databases, we have summarized the chronology of reports of white- and yellow-phenotype strains in various countries and looked for the temporal beginning of the epidemic and possible events of introduction to new territories (Fig.1; Paper 1). To unveil the population structure and to track genetic flow between populations, we developed a new highly sensitive subtyping scheme based on ten microsatellite markers. These markers have proven to be a useful tool for population genetic studies not only of T. benhamiae (Paper 1) but also of T. erinacei (Paper 4).

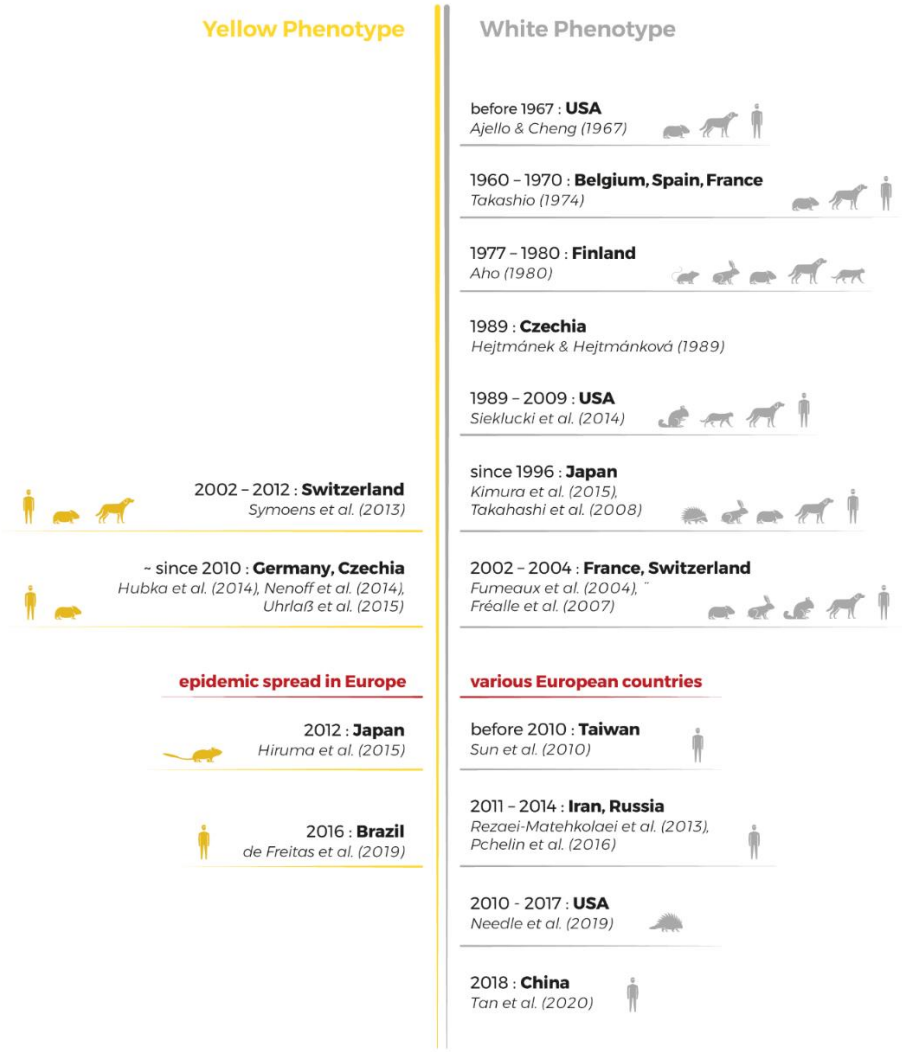

Fig. 1 Chronology of reports of *Trichophyton benhamiae* yellow and white phenotype strains from various countries, sorted according to the phenotypic characters of cultures reported by the authors and, in more recent studies, a combination of DNA sequencing and morphology (Paper 1).

TAXONOMY OF THE COMPLEX

In past literature, the white phenotype strains of *T. benhamiae* and *T. erinacei* were usually referred to as *Atrhoderma benhamiae* and *T. mentagrophytes, respectively*. The species *T. mentagrophytes* was broadly defined and included three biological species, *A. benhamiae*, *A. simii* and *A. vanbreuseghemii*. *Arthoderma benhamiae* was introduced as a sexual stage for part of *T. mentagrophytes* isolates, designated as *T. mentagrophytes* var. *granulosum*, and later also for strains of *T. mentagrophytes* var. *erinacei* (now *T. erinacei*) (Ajello & Cheng 1967). However, with introduction of molecular taxonomy, it become clear that the concept of pooling unrelated biological species under the name *T. mentagrophytes* was no longer sustainable. Therefore, *T. mentagrophytes* was split into several species complexes: *T. mentagrophytes* complex, *T. benhamiae* complex and *T. simii* complex, each encompassing several species (Gräser *et al.* 1999b). The concept of varieties in *T. mentagrophytes* complex which had been established based on morphology, had to be re-evaluated. Thanks to molecular methods it became apparent that individual morphotypes (varieties) were either superfluous, represented intraspecific variability or were composed of multiple species due to the phenotype similarity of unrelated taxa (Gräser *et al.* 1999b). From that point, the well-established status of variety gradually disappeared from the taxonomy of dermatophytes.

Arthroderma benhamiae , due to the position of its ex-type strain, forms a lineage closely related to *T. concentricum*, *T. eriotrephon, T. verrucosum* and *T. erinacei*, which all together formed a clade designated as the *Atrhoderma benhamiae* complex (Gräser *et al.* 1999a; Gräser *et al.* 1999b). Species *T. bullosum* was later confirmed as a well-defined species and added to the complex (Heidemann *et al.* 2010; Sitterle *et al.* 2012). After discontinuing the dual nomenclature *A. benhamiae* was transferred to the genus *Trichophyton* based on multi-locus phylogeny, while the genus *Arthroderma* was left for basal dermatophyte species with a predominant geophilic ecology (de Hoog *et al.* 2017). In total, the *T. benhamiae* complex (syn. *T. benhamiae* series) comprises five species: *T. benhamiae*, *T. concentricum*, *T. erinacei*, *T. eriotrephon* and *T. verrucosum* according to the last overview of dermatophyte taxonomy (de Hoog *et al.* 2017). Although de Hoog *et al.* (2017) did not include the species *T. bullosum* into the *T. benhamiae* complex, other studies classify it there (Lysková *et al.* 2015).

Before taxonomic revision carried out in this work, the naming of populations within *T. benhamiae sensu lato – i.e.,* taxonomic classification below the species level – was unsystematic, impractical, and confusing. Unsystematic naming on the example of *T. benhamiae* reflecting intraspecific phenotypic and genotypic variability and mating behaviour is schematically illustrated in Figure 2. Takashio (1974, 1977) examined mating compatibility of the ex-type strain of *T. benhamiae* (Ajello & Cheng 1967) from North America with strains collected in Europe and Africa and recognized two races based on the biological compatibility experiments an European-American race and an African race. Kano *et al.* (2008) found unique ITS and chitin synthase 1 genotypes which corresponded to the two races and assigned each genotype group a number; strains of European-American race are carriers

of Genotype I and the African race of Genotype II. Lasty, Genotype III "of *Arthroderma benhamiae*" has been set aside for Japanese hedgehog isolates (Kano *et al.* 2008). These strains, however, correspond to *T. erinacei* in its current concept and not to *T. benhamiae*. Furthermore, as mentioned in the previous chapter, two phenotypically different groups among strains of the European-American race (Genotype I) were recognized. The "yellow phenotype" strains of the European-American race with very unique morphology superficially resembling that of *Microsporum canis* are distinguished by various authors from "white phenotype" strains with similar morphology present in both European-American race and the African race (Symoens *et al.* 2013; Nenoff *et al.* 2014; Hiruma *et al.* 2015; Brasch *et al.* 2016). In addition, two groups were described in *T. benhamiae* based on phenotype and genotype and the presence of mating type gene idiomorphs. Group I was reported for most of the white phenotype strains from Europe and group II for yellow phenotype strains (Symoens *et al.* 2013). As an example, to illustrate the complexity of this subclassification, if we want to talk about *T. benhamiae* strains with white granular colonies from Europe, we can use the following designations: white phenotype strains of the European-American race of *T. benhamiae*, Group I of the European-American race of *T. benhamiae* or white phenotype strains of Genotype I of *T. benhamiae*. This is rather complicated. In the modern naming system proposed recently by de Hoog *et al.* (2023) also for dermatophytes, there are several possibilities for naming the taxa and clinically important genotypes/lineages in the complex which do not meet the requirements for species description. In the mentioned publication by de Hoog *et al.* (2023), they recommend either assigning numbers to genotypes or, for "closely related entities", using official nomenclatural categories such as subspecies or variety.

Therefore, we aimed to refine the naming of taxa in the complex and create a modern classification (Papers 1, 2 and 4). Our goal was for the new taxonomy to reflect the phylogeny/population structure and biology of the taxa, to be user-friendly and understandable for scientists and clinicians, and to comply with the International Code of Nomenclature for Algae, Fungi, and Plants (Turland et al. 2018). Although the species category is currently preferred for describing variability, in some cases it is still appropriate to use even lower classifications below the species level for populations exhibiting unique characteristics. For intraspecific classification, however, we did not want to follow Kano's numbering of T. benhamiae strains into three genotypes. We consider this classification unnatural because these genotypes are in fact lineages encompassing many genotypes and multiple species (at least T. benhamiae and T. erinacei). Re-introducing the rank of variety or subspecies to the taxonomy of dermatophytes seems to be a better option than confusing categories of genotypes, groups and morphotypes. In this regard, we also consider the variety rank to be a better option than other subspecific categories because this category was well established in dermatophytes in the past. In comparison with the concept of variety in the past, the current concept does not assign the status to different morphotypes of the same species, but it should be used to name clinically important or biologically unique lineages/populations that are not fully genetically separated from the species and

typically show incomplete lineage sorting when analysing multiple genetic markers (Paper 1). Our aim was to create a new taxonomic concept that is understandable for clinical mycologists through uniform and intuitive naming, while allowing taxonomists to find their way to a natural classification of species and lower-level diversity.

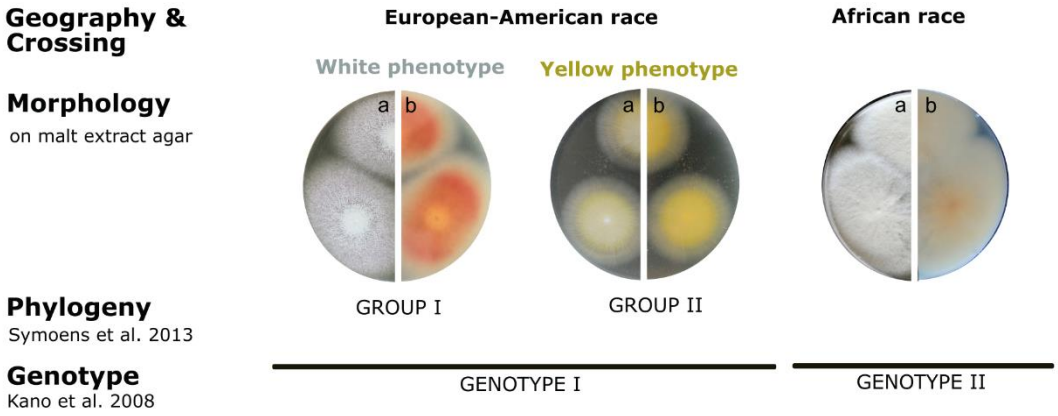

Fig. 2 A diagram illustrating the inconsistent naming of individual groups within *Trichophyton benhamiae*. Takashio (1974, 1977) identified a European-American race and an African race based on the biological compatibility experiments and the origin of isolates. Symoens *et al.* (2013) recognized a white (Group II) and yellow (Group I) phenotype based on morphology (macromorphology on Sabouraud dextrose agar) and ITS genotype. The characteristic morphology is most noticable in malt extract agar due to the red pigment produced by white phenotype strains, as seen on obverse (a) and reverse (b) of agar plates. Kano *et al.* (2008) identified Genotype I and II based on unique ITS and chitin synthase 1 genotypes.

SPECIES DELIMITATION ISSUES IN *TRICHOPHYTON*

The history of the study of dermatophytes is a long and thorny one. From the time Malmsten described the first *Trichophyton* in 1848 (Malmsten 1848), many species were introduced, but many soon became synonymized. The rise of cultivation techniques at the end of the 19th century resulted in a boom in the description of new dermatophyte species, followed by another, smaller wave due to the introduction of mating experiments in the 1960s and 1970s (de Hoog *et al.* 2017). Huge molecular studies at the turn of the millennium brought a decline in the number of accepted species (Gräser *et al.* 1999b; Gräser *et al.* 2000). The view of the species concept varied according to the methods available at the time, from phenotypical to biological compatibility detection to molecular. Finally, following the example of other organisms, a polyphasic approach combining various taxonomic methods was recommended for species delimitation in dermatophytes (Gräser *et al.* 2008). However, the application of the individual components of this concept is limited in many dermatophytes due to specific issues such as phenotype instability, high levels of clonality and incomplete lineage sorting. As a result, the "polyphasic" approach is commonly applied in a restricted form in taxonomic practice.

In this study, I used a polyphasic approach based on morphology, physiology, ecology, clinical picture, and molecular data including non-coding regions to resolve the taxonomy of the T. benhamiae complex (Papers 1, 2 and 4). However, to fully apply the polyphasic approach, we had to deal with many specific problems related to dermatophytes, which will be discussed in the following paragraphs.

PHYLOGENETIC CONCEPT

Progress in dermatophyte taxonomy is tightly linked to the development of new tools that enable us to effectively capture genetic variability. The key dermatophyte taxonomic phylogenetic studies published in the last three decades are mostly based on rDNA, especially the ITS region. Apart from ITS rDNA, some studies have included also protein-coding sequence markers such as *β*-tubulin (*tubb* or *tub2*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), actin, topoisomerase II and transcription elongation factor 1-α (*tef1-α*) genes (Kawasaki 2011; Rezaei-Matehkolaei *et al.* 2014; Mirhendi *et al.* 2015; de Hoog *et al.* 2017). However, some of these additional markers are not sufficiently variable in many primary pathogenic dermatophytes and are not easy to amplify compared to the ITS region, which remains a key taxonomic and diagnostic marker in dermatophytes. In addition, phenomena such as incomplete lineage sorting or occasional hybridization and introgression can further complicate species delimitation and identification by DNA sequence markers (Taylor *et al.* 2015; Steenkamp *et al.* 2018; Matute & Sepúlveda 2019) as shown by examples of species pairs *T. tonsurans*/ *T. equinum* and *T. mentagrophytes*/ *T. interdigitale* (Kandemir *et al.* 2020; Švarcová *et al.* 2023). The divergence between these evolutionarily young species/populations may be hidden when using some classical protein-coding phylogenetic markers, and the use of more sensitive population-genetic markers may be necessary. The use of more sensitive genotyping techniques, as well as non-coding highly variable markers in dermatophyte taxonomy and population genetics is essential due to the high degree of clonality in many primary pathogenic species. Genotyping techniques are not only used to uncover population structure, but they are often employed to gain insight into the dynamics of disease transmission, determine the source and routes of infections, confirm or rule out outbreaks, recognize virulent strains and assess regional and global changes in genotype patterns and evaluate the effectiveness of control measures (Ranjbar *et al.* 2014). Other common issues with dermatophytes involve the differentiation of relapse versus reinfection and the determination of whether the infection is caused by one or multiple strains, as well as if genotypes differ in their clinical manifestation. Many methods have been developed for the subtyping of dermatophytes, yet a significant number of them are now obsolete, and their utility is often limited due to poor reproducibility or unsatisfactory strain differentiation (Abdel-Rahman 2008; Mochizuki *et al.* 2017; Hubka *et al.* 2018b). MLST typing approaches have been widely applied to many fungal pathogens (Meyer *et al.* 2009; Debourgogne *et al.* 2012; Bernhardt *et al.* 2013; Maitte *et al.* 2013; Prakash *et al.* 2016) and are emerging as an important tool for dermatophyte population studies (Kandemir *et al.* 2020). However, the currently available loci usually lack sufficient discriminatory power to study the population structure of *Trichophyton* and *Microsporum* species in detail, or even to differentiate species, leading to ambiguities in the definition of species boundaries and species identification (de Hoog *et al.* 2017; Aneke *et al.* 2021). Neutrally evolving or noncoding DNA regions, such as microsatellites, introns and intergenic spacers, which accumulate mutations more rapidly, have been shown to reveal the evolutionary trajectories of primary pathogenic dermatophytes more

effectively (Gräser *et al.* 2008; Mochizuki *et al.* 2017; Hubka *et al.* 2018b). Furthermore, detailed genomic, epigenetic and multigene phylogenetic studies on a large number of samples can resolve delimitation issues between these species in the future (Pchelin *et al.* 2019; Singh *et al.* 2019). Single-nucleotide polymorphism detection by whole-genome sequence typing was used to infer the genetic relatedness of the global population of *T. rubrum* on a limited dataset of strains (Persinoti *et al.* 2018; Zheng *et al.* 2020; Tang *et al.* 2021). This approach will ultimately become one of the methods of choice in the future with decreasing costs (Hadrich & Ranque 2015). Currently, sequencing of ITS rDNA and population genetic markers such as microsatellites (Kaszubiak *et al.* 2004; Gräser *et al.* 2007; Pasquetti *et al.* 2013) or mixed-marker approaches (Abdel-Rahman *et al.* 2010; Aneke *et al.* 2021) offer higher discriminatory power in subtyping of primary pathogenic dermatophytes compared to MLST approaches. Microsatellite markers are still among the most effective tools available for the subtyping of dermatophytes and allow the analysis of large numbers of strains with high reproducibility. Typing schemes have been developed for a limited number of species, including *T. rubrum* (Gräser *et al.* 2007), *Nannizzia persicolor* (Sharma *et al.* 2008) and *M. canis* (Sharma *et al.* 2007; Pasquetti *et al.* 2013; Aneke *et al.* 2021). Polymorphisms in *T. benhamiae* (European-American race) were previously investigated by the RFLP analysis of the NTS region, which produced 11 different patterns in 46 isolates; this method successfully confirmed laboratory-acquired infections as well as familial outbreaks transmitted from pets (Mochizuki *et al.* 2002; Takeda *et al.* 2012).

It was crucial for our study to determine gene flow between populations/lineages of T. benhamiae sensu lato, as well as the entire T. benhamiae complex, in order to determine species limits and to address the emergence of yellow phenotype strains of T. benhamiae in Europe. Therefore, we developed a microsatellite typing scheme consisting of ten variable markers in addition to the multilocus sequence typing scheme consisting of the ITS rDNA region (ITS1-5.8S-ITS2 cluster), partial gapdh, tubb and tef1-α genes. Initially, the typing scheme was developed for taxa from the T. benhamiae clade (Papers 1 and 2). Due to the close relatedness of the members in the complex, seven out of ten microsatellite markers could also be used in T. erinacei (Paper 4). A Bayesian model-based clustering algorithm implemented in STRUCTURE was used to determine how many groups were included in the datasets of the T. benhamiae clade and T. erinacei.

In the multilocus phylogenetic trees (Maximum likelihood and Bayesian inference methods), members of the T. benhamiae complex (n = 339 strains) were resolved into three major monophyletic clades (Paper 1, Fig. 3). The T. benhamiae clade contained T. concentricum and the European-American race of T. benhamiae. The isolates of the European-American race did not form a monophyletic lineage and was paraphyletic with respect to T. concentricum. In addition, T. benhamiae clade strains were segregated into several well-supported subclades numbered 1-6 (Fig. 3): subclade 1 included T. concentricum, subclade 2 comprised the ex-type strain of T. benhamiae including other North American white phenotype strains and yellow phenotype epidemic strains; remaining white

phenotype strains formed subclade 5 and 6 (Paper 1). Furthermore, after comparing our ITS rDNA sequences with available sequences in GenBank, we additionally identified two potentially new taxa that formed independent lineages separated from each other and other taxa in the T. benhamiae clade – subclades 3 and 4 (Paper 2; Fig 3).

A total of 43 microsatellite genotypes among T. benhamiae clade isolates formed eight clusters (Fig. 4; Fig.6 in Paper 1; Fig. 5 in Paper 2). In contrast to the multigene phylogeny, population-genetic analysis based on microsatellite data revealed additional structure in the T. benhamiae clade. *Specifically, in subclades 1-2, T. concentricum and yellow phenotype strains were clearly separated from white phenotype strains of North American origin (Fig. 4). In addition, a high variability was detected among North American strains by microsatellite data in contrast to the highly clonal yellow phenotype strains. While the multigene phylogeny assigned subclades 5 and 6 to the vicinity of 3 and 4 (Fig. 3), microsatellite data rather showed their relatedness with North American strains of subclade 2 (Fig. 4; Fig. 5 in Paper 2).*

The T. erinacei clade comprised three species: T. erinacei, T. verrucosum and T. eriotrephon. Follow-up multigene phylogenetic analysis on a larger dataset of T. erinacei strains (n = 161, Paper 4) including samples collected from free living hedgehogs, pet hedgehogs and their owners, revealed five multilocus genotypes. Interestingly, these genotypes were not completely separated from each other as was evident from the highly incongruent single-gene phylogenies which is unusual in pathogenic dermatophytes. A total of 10 genotypes distributed in two clusters were revealed among T. erinacei isolates based on the STRUCTURE algorithm applied to microsatellite data (Fig. 6 in Paper 1; Fig. 5 in Paper 4). These two clusters were not clearly separated and there was still gene flow between them as evidenced from the incongruences between phylogenies constructed from sequence or microsatellite data. Therefore, T. erinacei was supported as a single monophyletic species with notable intraspecies variability in genotype and phenotype (Fig. 3 in Paper 4).

The T. bullosum clade contained isolates of the African race of A. benhamiae and T. bullosum; it was thus clear that the African race of A. benhamiae was a new species more closely related to T. bullosum than to T. benhamiae sensu stricto with which it had formerly been associated (Paper 1).

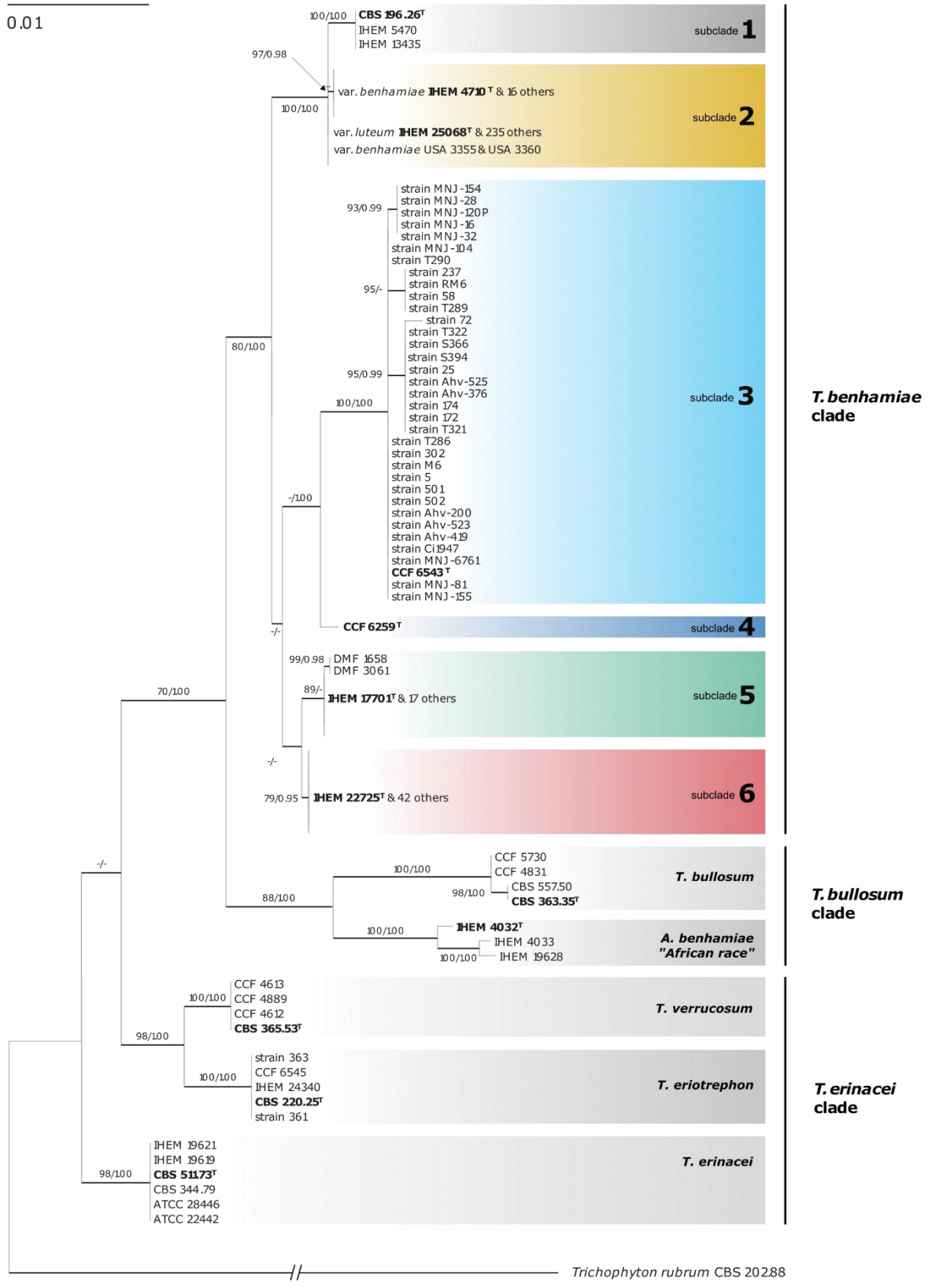

Fig. 3 A best-scoring maximum-likelihood tree (*gapdh*, *tubb*, ITS rDNA, and *tef1-α*) showing three clades in the *T. benhamiae* complex and six subclades (numbered) in the *T. benhamiae* clade.

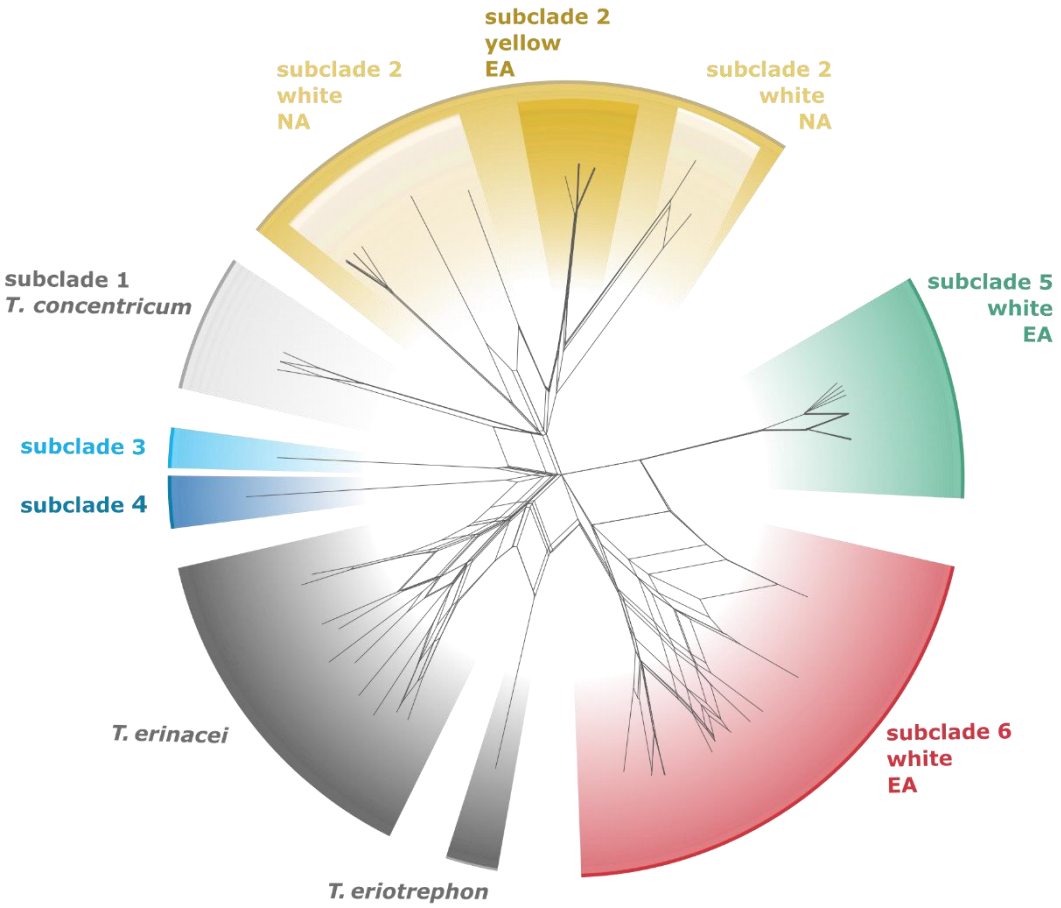

Fig. 4 A phylogenetic network was constructed using microsatellite data from 483 strains, utilizing seven microsatellite loci. The network revealed the presence of six subclades, numbered, within the *T. benhamiae* clade, as well as the species *T erinacei* and *T. eriotrephon*. The NeighborNet network was created using FAMD 1.3 software and visualized in SplitsTree 4.13 utilizing the Jaccard index-based distance matrix. Different colours were used to indicate the classification of strains into species and subclades. The labels of each subclade indicate the geographic origin of the strains (NA refers to North America, EA refers to Eurasia), as well as their belonging to one of the phenotypes (white and yellow) of the *T. benhamiae*.

PHENOTYPIC SPECIES DEFINITION IN DERMATOPHYTES

Appearance of colonies on selected microbiological media, growth rate, growth at 37°C, urease activity, growth on Trichophyton T1-T7 agars, hair perforation, and various micromorphological features (dimensions and features of conidia, conidiophores, ascospores and ascomata) are phenotypic criteria that perform relatively well in routine diagnostics of some dermatophyte species but fail in some other species. With the emergence of strains resistant to antifungal agents, much attention is also being paid to the different susceptibilities of different species to these agents. For example, in one clonal lineage of *T. mentagrophytes*, the higher sensitivity of some strains led to the description of the lineage as a new species, *T. indotineae (Kano et al. 2020)*. However, this population is now found to contain both susceptible and resistant strains. Like other phenotypic traits, susceptibility to antifungal agents seems to be highly variable. As in other fungi, some unrelated dermatophyte taxa show phenotypic similarities and their reliable identification is difficult or impossible without molecular methods (Heidemann *et al.* 2010; Summerbell 2011; Lysková *et al.* 2015; Uhrlaß *et al.* 2018; Su *et al.* 2019; Kandemir *et al.* 2020). Additionally, phenotype frequently changes depending on the length or preservation due to the rapid degeneration of important culture characteristics and changes in their physiology (de Hoog *et al.* 2017). Consequently, it can be difficult to maintain and reproduce phenotypic characters over decades for the purposes of taxonomic studies. The change in phenotype can be triggered by other stress factors besides ageing, e.g., UV light or antimycotic exposure (de Hoog *et al.* 2017). In stressed strains, the growth rate increases or decreases, the texture of the colony becomes fluffy, and microconidia are no longer produced and may be replaced by macroconidia or chlamydospore formation. To make a conclusion about the existence of a particular taxon based only on a minimalist dataset of strains, for example, only one locality, we run the risk that such a conclusion may be wrong due to pleomorphism. As an example, species *T. proliferans* was shown to be an atypical strain of *T. erinacei* undergoing ageing (English & Stockdale 1968). Similarly, *T. mentagrophytes* undergoing stress tends to show morphology typical for *T. mentagrophytes var. interdigitale* (Švarcová et al. 2023). As a conclusion, phenotypic criteria are not given much attention in taxonomic assessment. However, if included and evaluated, a weak correlation between genotype and phenotype is usually noticeable in most studies on dermatophytes (Heidemann *et al.* 2010; Lysková *et al.* 2015; Uhrlaß *et al.* 2018; Kandemir *et al.* 2020; Tang *et al.* 2021; Švarcová *et al.* 2023).

To identify stable characters for use in the taxonomy of the T. benhamiae complex, we collected a considerable number of phenotypic measurements which were evaluated later using statistical methods. In addition to basic morphological features (colony appearance, size of spores, presence, absence, or abundance of some micromorphological features) and basic physiological characteristics (growth rates at different temperatures), we also employed MALDI-TOF mass spectrometry analysis to aid in the

translation of taxonomic conclusions to clinical practice. We decided not to include some other physiological and biochemical tests such as urease activity and hair perforation as they proved to be rather unstable among strains of well-defined species and their importance decreased in the molecular era (Robert & Pihet 2008; Tang et al. 2023).

The analysis, using only data for the T. benhamiae clade, showed that there were statistically significant differences between subclades according to any combination of characteristics, including conidia size and growth rates (Paper 1). The strains of subclade 3, T. concentricum, and yellow phenotype strains of subclade 2, were characterized by the slowest growth on all media and at all tested temperatures (Paper 1, Fig. 11). No sporulation was observed in the T. concentricum strains. Poor sporulation, the absence of macroconidia and spiral hyphae and production of intense pigments were typical for the yellow phenotype strains from subpopulation 2 (intense yellow) and subpopulation 3 (intense diffuse brown pigment). All four remaining subclades from the T. benhamiae clade (previously referred to as white phenotype strains of T. benhamiae) produced whitish colonies with a brownish, red-brown, or red colony reverse, and they produced both micro- and macroconidia and. North American strains from the subclade 2 grew more rapidly at 25 C than the members of the other subclades and exhibited larger microconidia on average.

Outside of the T. benhamiae clade, slow growth and poor sporulation were typical for species T. bullosum and T. verrucosum which also showed restricted sporulation while the remaining species grew significantly faster and more extensively sporulated (Papers 1 and 2). Other morphological features such as the presence and absence of macroconidia, chlamydospores and spiral hyphae may also be important in specific cases. Although these structures can be observed in most species, they can differ in the quantities in which they are produced relative to other structures. Among T. erinacei strains, slight differences were found between strains of two different microsatellite clusters in conidia size; however, we found no other differences in the micro- and macromorphology which could be linked to MLST genotypes or microsatellite clusters (Paper 4).

A pilot MALDI-TOF MS analysis in T. benhamiae clade species was able to identify specific peaks for all subclades in the T. benhamiae clade (Paper 1). Finally, some taxa (subclades 2, 3, 5 and 6, T erinacei, and T eriotrephon) have also been tested for susceptibility to various antifungals. No significant differences in susceptibility were found between species from the complex and all strains were assessed as susceptible (Paper 5). However, upon closer examination of the T. erinacei strains we detected statistically significant differences in susceptibility to ciclopirox, terbinafine, and tavaborole between both microsatellite clusters (Paper 4).

ECOLOGICAL CONCEPT

Geographical barriers and host specificity are among the mechanisms that shape dermatophyte speciation through an allopatric speciation process (Weitzman & Summerbell 1995). Therefore, it makes biological sense to include geographical distributions and host preferences in taxonomic considerations. While many dermatophytes have a global distribution, some species are restricted to specific geographical areas, this is well documented in species from the *T. rubrum* complex and *Microsporum* species (Kaszubiak *et al.* 2004; Hayette & Sacheli 2015; Packeu *et al.* 2020). Pathogenic dermatophytes have complex geographic distributions that mirror those of their hosts, and are influenced by various factors, such as environmental conditions (humidity, temperature) and socioeconomic and behavioural factors (Hubka *et al.* 2018a). However, the distribution of some dermatophytes is restricted to only part of the host's distribution range; for instance, the distribution of some anthropophilic species (*T. concentricum*, *T. violaceum*, *T. soudanense* and *T. tonsurans*) is geographically restricted or uneven compared to the distribution of their human host. Therefore, in addition to host preference, socio-economic, cultural, hygienic and environmental factors likely play a role in dermatophyte spread (Havlickova *et al.* 2008; Moulíková *et al.* 2023). Although some dermatophytes, such as *T. erinacei*, seem to be strongly host specific, the host range of other species may be much wider. Giving host specificity more emphasis in taxonomic assessment may thus lead to wrong conclusions and oversplitting of species; examples of this are highly discussed species pairs, such as *T. equinum*/*T. tonsurans* and *T. mentagrophytes*/*T. interdigitale*. Zoophilic species, *T. mentagrophytes* and *T. equinum*, were established partly as opposites of their anthropophilic counterparts *T. interdigitate* and *T. tonsurans* although, according to some other authors, they are considered a single species with two insufficiently separated populations and host spectrums involving both humans and animals (Kandemir *et al.* 2020; Švarcová *et al.* 2023). The knowledge about distribution and host affinity of almost all zoophilic dermatophyte species is still fragmentary. Our knowledge of the ecology of these pathogens is mostly limited to domestic animals and pets, and little is known about reservoirs in wild-living animals; thus, almost any ecological hypothesis about the species origin is based on very incomplete data and needs to be refined by future research. Additionally, it must be considered that both the host spectrum and the distribution are dynamic and change over time due to international travel, migration, and animal transport, which erases, to a large extent, the original geographic areas of distribution. Sequence databases such as GenBank provide the most reliable information on species distribution; however, there are main limitations due to the lack of epidemiological and DNA data from America, many Asian countries and Africa. Databases of environmental samples such as GlobalFungi contain millions of environmental sample reads, mainly from soil and root samples, all over the world (Větrovský *et al.* 2020). However, they have not yet contained any sequences of *Trichophyton* species, confirming that zoophilic and anthropophilic dermatophytes are almost absent in the soil environment. Nevertheless,

they can still harbour in microhabitats associated with their hosts in the environment, which have not been sampled in any studies yet.

The application of clinical data for species delimitation is at the centre of debate for some species. Clinical data can be useful in taxonomy, if used correctly and based on a large dataset of strains. Clinical manifestation and localization of infection may reflect the actual ecology of the species (e.g., to distinguish anthropophilic and zoophilic nature of species) and mode of transmission between hosts (Hayette & Sacheli 2017).

To understand the global distribution of the T. benhamiae complex populations, we analysed several hundred ITS rDNA sequences deposited in GenBank. We focused mainly on the T. benhamiae clade (Paper 1). For most of the species in the complex, we did not have enough data to make a conclusive conclusion about their distribution. Extensive epidemiological studies, supported by ITS rDNA data, have been conducted mostly in European countries, Iran, and Japan, and on a limited basis in several countries scattered unevenly around the world. Epidemically spreading yellow phenotype strains of T. benhamiae subclade 2 have their main distribution areas in Europe in guinea pigs whereas white phenotype strains of the same subclade seem to be distributed only in North America, mostly in dogs. However, the reservoir of North American strains may be hidden in wild living hosts, e.g., the North American porcupine (Erethizon dorsatum). Free-living hosts are just assumed due to the high genetic variability of North American strains and few documented cases of infection of North American porcupines (Takahashi et al. 2008; Needle et al. 2019). Trichophyton concentricum is typically found in tropical regions of Oceania, Southeast Asia, and Central and South America. White phenotype strains of T. benhamiae clade subclades 5 and 6 have their main distribution in Europe in guinea pigs. In addition, strains of subclade 5 are responsible for the majority of human dermatophytosis due to T. benhamiae in Japan with a predominant reservoir in rabbits (Paper 1).

In addition, several sequences of isolates originating in Czech Republic and Iran (strains of the T. benhamiae clade, subclades 3 and 4) could not be linked to their respective species in the complex, which led to a complementary study to clarify their relationship to already published species (Paper 2). Two newly discovered subpopulations (3 and 4) in the T. benhamiae clade were documented by us for the first time. Strains of subpopulation 3 were isolated from more than 30? cases of human and one cat dermatophytosis in Iran. The only strain of subclade 4 was isolated from human dermatophytosis contracted probably from a dog in the Czech Republic.

BIOLOGICAL CONCEPT

The assessment of species boundaries through mating experiments, which reveal biological compatibilities, played an important role in the delimitation of many early species and the discovery of their sexual stages. This approach, based on the Biological Species Concept (BSC), is generally highly applicable in geophilic dermatophytes (Dawson & Gentles 1962; Stockdale 1964; Padhye & Carmichael 1972; Choi *et al.* 2012; Hubka *et al.* 2015; Moulíková *et al.* 2023). By contrast, the results of biological compatibility assessments can considerably differ from the currently accepted species boundaries in anthropophilic and zoophilic dermatophytes. These species are evolutionarily younger, and their phylogenetic divergence occurred earlier than the development of reproductive barriers. As a result, various primary pathogenic *Trichophyton* species show an *in vivo* ability to hybridize with unrelated species (Kawasaki *et al.* 2009; Anzawa *et al.* 2010; Kawasaki *et al.* 2010; Kawasaki 2011). However, it is highly unlikely that this kind of hybridization occurs naturally due to the different ecological niches (hosts). The results of *in vitro* mating assays therefore cannot be fully compared to a natural scenario. Additionally, the ratio of mating-type gene idiomorphs is usually extremely imbalanced, or one idiomorph is missing, in the majority of primary pathogenic dermatophytes (Metin & Heitman 2017; Kosanke *et al.* 2018). This fact further limits or even prevents the possibility of using BSCs in the delimitation of these species. The ancestors of many currently recognized pathogenic dermatophytes were likely sexually reproducing geophilic species and zoophilic species on free-living mammals, such as those found in soil surrounding burrows, with balanced ratios of opposite mating type individuals (Gräser *et al.* 2006; Summerbell 2011). Introduction to new areas and/or adaptation to a new host is a unique event in the evolution of many anthropo- and zoophilic dermatophytes, resulting in the extinction of one mating partner in the entire population of these species. Only some offshoots of ancestral sexual dermatophytes probably maintain ongoing populations and follow independent evolutionary trajectories towards speciation (Gräser *et al.* 2006). Alternatively, the extinction of one MAT gene in a population of dermatophytes may be caused by the preferential spread of strain(s) exhibiting an advantageous combination of alleles associated with higher virulence/transmission potential. Such a successful genotype may be significantly dominant in conditions with almost exclusive asexual transmission and may displace other genotypes. This situation is very likely to lead to an imbalance in the MAT gene ratio or even the loss of one MAT gene in the population as seen in many pathogenic dermatophytes, e.g., *T. verrucosum* (Kosanke *et al.* 2018). Almost complete extinction of strains belonging to one mating type is, for instance, observed in *M. canis* (Sharma *et al.* 2007; Kosanke *et al.* 2018). In addition, different levels of virulence linked with mating-type idiomorphs have been repeatedly documented in fungal pathogens (Yue *et al.* 1999; Chang *et al.* 2000; Cheema & Christians 2011). The increased virulence of one of the mating types can also be expected in dermatophytes.

Due to the specific problems mentioned above, we did not focus on the presence of reproductive barriers between groups by conducting crossing experiments, as it has been shown in previous studies that

prezygotic reproductive barriers are incomplete. To determine the level of clonality and to detect possible recombination events inside and outside populations, we needed to detect gene flow between populations based on genetic markers (see subchapter "Phylogenetic concept") and the distribution of mating types in populations. We determined mating type idiomorphs for all strains in the study and calculated various population characteristics (gene and genotype diversity, effective number of genotypes, index of association, pairwise fixation index and a coefficient of genetic differentiation) (Paper 1 and 4). All the indexes examined confirmed that clonal reproduction is the dominant mode of dissemination in the T. benhamiae complex. The mating type ratio was extremely unbalanced, typically with just one mating type present in strains of a population. The mating type MAT1-1-1 was present in all strains of T. bullosum, subclade 1, 5 and yellow phenotype strains from subclade 2. The mating type MAT1-2-1 was present in all strains of T. verrucosum and was dominant in subclade 6 and North American strains of subclade 2. Both mating type was present in African race of A. benhamiae and the mating type ratio was almost balanced in T. erinacei, but strongly unbalanced in both T. erinacei microsatellite clusters.

Finally, based on all population characteristics, recombination is absent in almost all populations except for T. benhamiae clade subpopulation 6 (Paper 1) and T. erinacei (Paper 4). Slightly contradictory results were obtained for T. erinacei: the hypothesis on random mating was rejected (though not at a significance level) but signs of sexual reproduction/recombination in the population were observed in the collected data (both mating type gene idiomorphs in the population, incongruences between DNA sequence data and microsatellite data, and level of intraspecific genetic variability,). However, rejecting the hypothesis of random mating does not mean that there are no occasional recombination events between T. erinacei strains, rather, it reflects that the isolates examined in the present study spread clonally between the hosts from which they were isolated: isolates belonging to one of two main populations spread clonally between pet African hedgehogs, while the second population spread predominantly between Erinaceus wild hedgehogs (Paper 4). Although wild hedgehogs are in close contact with soil where sexual reproduction should take place and where genetic variability is expected to be generated by recombination, we detected only very low genetic variability among 115 isolates from European hedgehogs from France (Papers 3 and 4). This indicates that sexual reproduction and de novo infection of hedgehogs from soil is a rare event and that clonal spread secured by horizontal spread from individual to individual strongly dominates (Paper 4).

NEW TAXONOMY OF THE *TRICHOPHYTON BENHAMIAE* COMPLEX

A polyphasic approach combining independent phylogenetic and population-genetic analyses with phenotypic features and ecological data helped to overcome most obstacles to species delimitation in the *T. benhamiae* complex. The initial hypotheses about the possible division of the complex, postulated based on phylogeny, were in line with population-genetic analyses and phenotypic characteristics such as conidia size and shape, colony morphology and size. These features were shown to be useful supportive taxonomic characters when observed on a large set of freshly obtained samples, with morphological differences being observed even in close taxa. In contrast, no significant differences were found in the antifungal susceptibility of *T. benhamiae* complex members as they were evaluated as sensitive to all tested antifungals (Paper 5). The only exception was *T. erinacei* where significantly different antifungal susceptibilities were observed between two T. erinacei populations adapted to different hosts (Paper 4).

To overcome the problem of low discriminatory power of DNA sequences, we had to come up with a more sensitive molecular method of reconstructing phylogenetic relationships than the commonly used markers. Population genetic analysis using our newly developed microsatellite typing scheme was able to better capture the gene flow and separate some biologically unique lineages that were not distinguished by multigene phylogeny, e.g., white and yellow phenotype strains of subclade 2 (Papers 1, 2 and 4).

Polyphasic approach supported the recognition of eleven taxa in the *T. benhamiae* complex, including five new species and one new variety (Papers 1, 2 and 4; Fig. 5 and Fig. 6). The number of species in the complex has thus almost doubled compared to the situation before the revision (six species). The most changes occurred in the *T. benhamiae* clade, where we introduced a new variety and four new species alongside two already existing species, *T. benhamiae* and *T. concentricum* (Papers 1 and 2). We found notable ecological and morphological differences between yellow phenotype and white phenotype strains of *T. benhamiae* in subclade 2. Yellow phenotype strains, which were almost exclusively associated with guinea pigs in Europe, can be identified by their slow growth on all media at all temperatures and uniform phenotype (yellow pigmentation and absence of macroconidia. White phenotype strains were found only in the USA (mostly dogs) and exhibited strikingly different colonies with a brown to red-brown reverse side, produced macroconidia and larger microconidia than yellow phenotype strains. The differentiation of yellow phenotype strains has clinical relevance, which is why we decided to reassign yellow phenotype strains as a variety, *T. benhamiae* var. *luteum*. Closely related white phenotype strains of subclade 2 comprising are named *T. benhamiae* var. *benhamiae* (automatically generated variety). We chose this conservative approach with varieties rather than the proposals of a new species because of the impossibility of distinguishing this entity using available DNA sequence markers, which is currently the standard in fungal taxonomy.

Despite some overlap in ecology and morphology of "white phenotype" strains from subpopulations 5 and 6, reliable differentiation was only possible by means of molecular methods (ITS and *gapdh* gene sequences, microsatellite markers, MALDI-TOF MS). In addition, we detected complete isolation of these two populations in the molecular data and this led us to introduction of two new species, *T. europaeum* and *T. japonicum* (Paper 1). The members of subclades 3 and 4 were proposed as new species, *T. spiraliforme* and *T. persicum*, with a poorly resolved position in the *T. benhamiae* clade. *Trichophyton persicum* was characterized by limited distribution (strains were isolated exclusively in Iran), poor sporulation, restricted growth at 37 °C and a brown diffuse pigment. Its sister species, *T. spiraliforme*, produced large amounts of spiral hyphae on all tested media (Paper 2). Part of our taxonomical conclusions done in the *T. benhamiae* clade and published in Paper 1 were supported in a follow-up study (Baert et al. 2021) with the exception of the separation of *T. japonicum* and *T. europaeum*.

Outside the *T. benhamiae* clade, we found significant intraspecific variability within *T. erinacei* (Papers 3 and 4). Although the process of speciation into two lineages according to the different hosts is ongoing, there is still evident gene flow between them, and phenotypic differences are rather small. Therefore, we present *T. erinacei* as a single species, with notable intraspecies variability in genotype and phenotype (*Paper 4*). Additionally, we introduced a species name, *T. africanum*, for strains previously referred to as the "African race of *A. benhamiae*" (*Paper 1*).

The sexual process in *T. benhamiae* complex members is likely rare or absent as evidenced by the lack of one MAT gene idiomorph in many species or extremely low level of intraspecific genetic variability revealed through MLST or microsatellite typing (Papers 1, 2 and 4). Recombination is usually considered primarily in populations/species widespread in free-living animals. However, our data from wild hedgehogs indicated that sexual reproduction in *T. erinacei* and de novo infection of hedgehogs from soil are probably rare events and that clonal horizontal spread strongly dominates in natural populations (Paper 4).

Due to the availability of epidemiological data, with only part of Western and Middle Europe having sufficient coverage from extensive epidemiological studies, we have only fragmentary knowledge about the distribution of *T. benhamiae* complex taxa. However, it is apparent from the data collected in this study and published in databases that the distribution of some species has its own geographic pattern. Animal trade and transport may play an important role in spreading the species to new geographical areas, and we hypothesize that it probably caused the introduction of yellow phenotype strains (*T. benhamiae* var. *luteum*) to Europe and Japan (Paper 1). While the introduction of this variety to Japan can be traced to two well-documented examples, we have no evidence of how these strains were introduced to Europe after 2000. Based on relationships of strains at the phylogenetic and population-genetic levels, we assume the origin of *T. benhamiae* var. *luteum* in North America where closely related strains of *T. benhamiae* var. *benhamiae* have their natural reservoir in free-living animals (Takahashi et al. 2008; Needle et al. 2019). However, host spectrum of *T. benhamiae* var. *benhamiae*

can be broader and may include members of the Canidae family, as evidenced by repeated isolation from dogs (Ajello & Cheng 1967; Sieklucki et al. 2014) and a patient who had contact with a fox (Tan et al. 2020). An extensive sampling in North America is crucial for clarifying the population structure and the origin of epidemic strains.

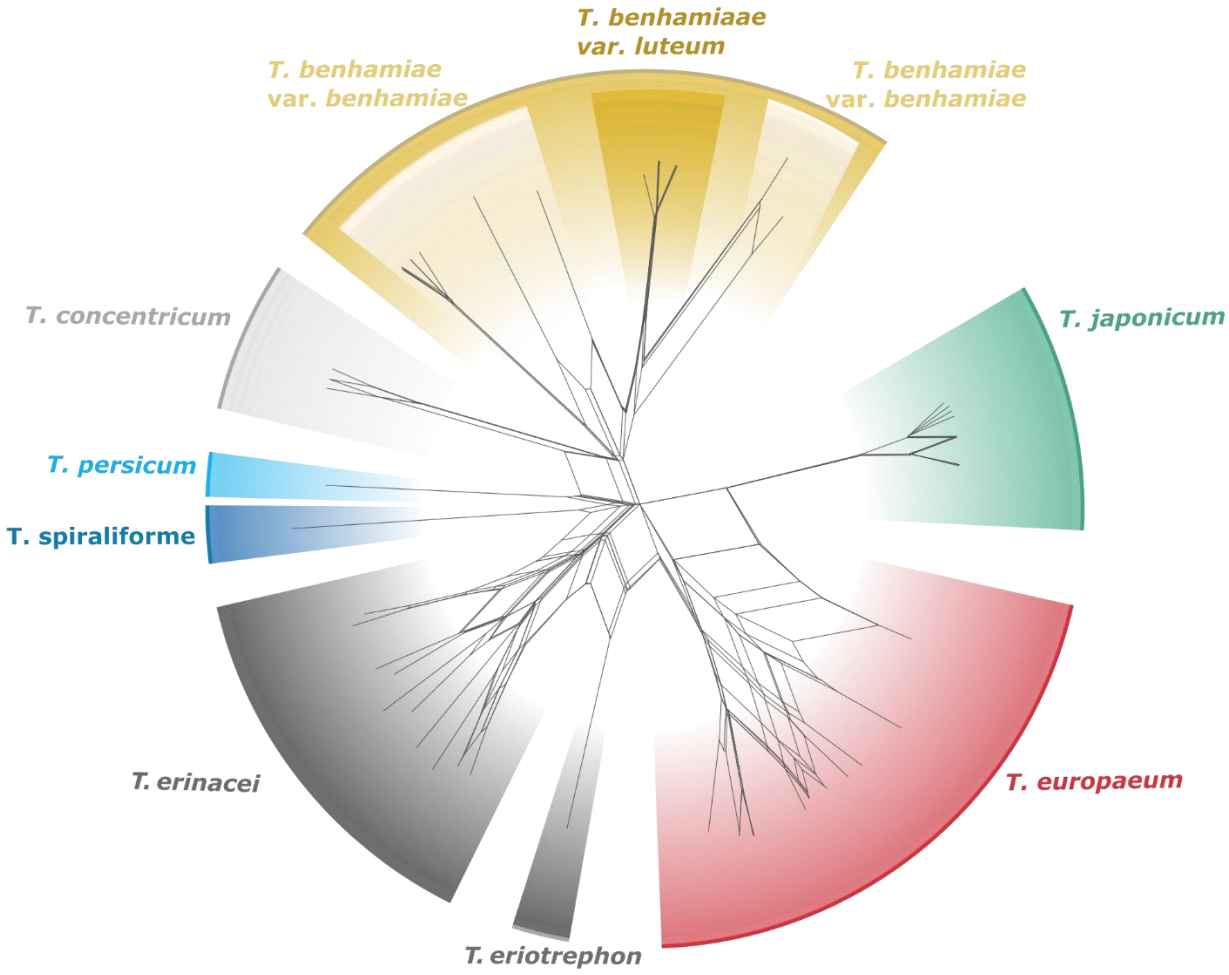

Fig. 5 SplitsTree phylogenetic network was constructed using microsatellite data from 483 strains, with seven microsatellite loci. The network revealed the presence of four new species and two new varieties within the *T. benhamiae* clade. The NeighborNet network was created using FAMD 1.3 software and visualized in SplitsTree 4.13 utilising the Jaccard index-based distance matrix. Different colours were used to indicate the classification of strains into species and varieties.

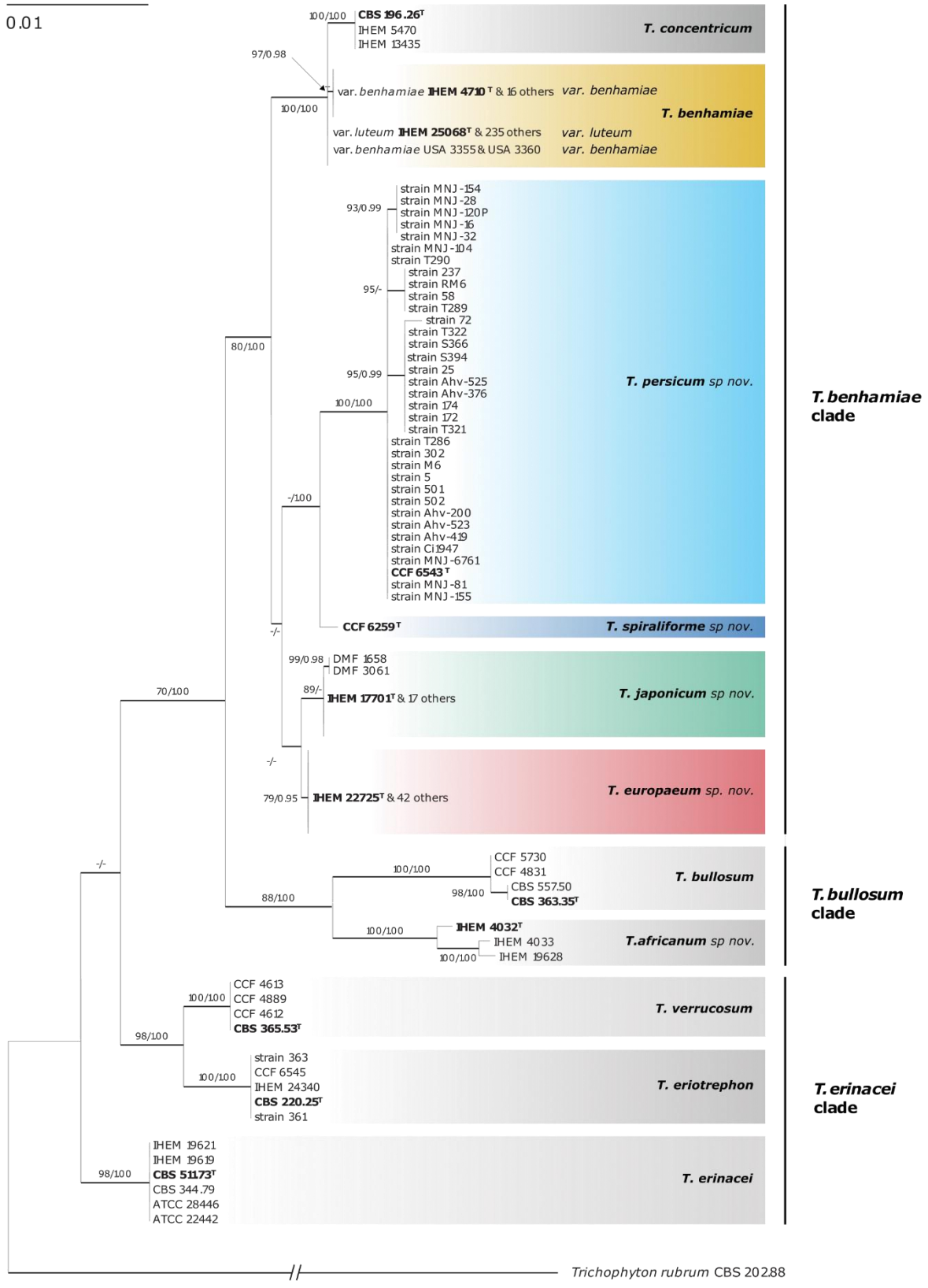

Fig. 6: A best-scoring maximum-likelihood tree (*gapdh*, *tubb*, ITS rDNA, and *tef1-α*) showing four new species and two new varieties in the *T. benhamiae* clade.

CONCLUSION

The prevalence and spread of emerging pathogens from the *T. benhamiae* complex require close monitoring, particularly because infection rates in the principal hosts - guinea pigs, hedgehogs, rabbits, dogs, porcupines, and other mammal pets and livestock animals – can be high, yet often without significant symptoms. The new taxonomic classification and improved accessibility of largely comparable data (microsatellites, SNPs and DNA sequences) will be important for understanding the global epidemiology of these emerging dermatophytes and for monitoring changes in genotype spectra on a global scale. This new typing scheme is currently the most powerful tool for the subtyping of *T. benhamiae* complex species; it is easy to use and cost-effective due to its multiplex design (one PCR reaction). To facilitate the implementation of these taxonomic changes into practice, in accordance with One health approach, we have prepared a identification manual for species identification in clinical practice based on phenotypic and molecular methods (Fig. 4). The newly proposed taxonomic classification and typing scheme will help to evaluate the results of preventive measures and interventions and is a basic prerequisite for the preparation of high quality epidemiological and population-genetic studies.

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PAPER 1

One Health approach to infections caused by *Trichophyton benhamiae* complex pathogens

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

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ABSTRACT

Species of the *Trichophyton benhamiae* complex are predominantly zoophilic pathogens with a worldwide distribution. These pathogens have recently become important due to their epidemic spread in pets and pet owners. Considerable genetic and phenotypic variability has been revealed in these emerging pathogens, but the species limits and host spectra have not been clearly elucidated. In this study, we used an approach combining phylogenetic analysis based on four loci, population-genetic

data, phenotypic and physiological analysis, mating type gene characterization and ecological data to resolve the taxonomy of these pathogens. This approach supported the inclusion of nine taxa in the complex, including three new species and one new variety. *Trichophyton benhamiae* var. *luteum* var. nov. ("yellow phenotype" strains) is currently a major cause of zoonotic tinea corporis and capitis in Europe (mostly transmitted from guinea pigs). The isolates of the "white phenotype" do not form a monophyletic group and are segregated into three taxa, *T. benhamiae* var. *benhamiae* (mostly North America; dogs), *T. europaeum* sp. nov. (mostly Europe; guinea pigs), and *T. japonicum* sp. nov. (predominant in East Asia but also found in Europe; rabbits and guinea pigs). The new species *T. africanum* sp. nov. is proposed for the "African" race of *T. benhamiae*. The introduction to new geographic areas and host jump followed by extinction of one mating type gene have played important roles in the evolution of these pathogens. Due to considerable phenotypic similarity of many dermatophytes and phenomena such as incomplete lineage sorting or occasional hybridization and introgression, we demonstrate the need to follow polyphasic approach in species delimitation. Neutrally evolving and noncoding DNA regions showed significantly higher discriminatory power compared to conventional protein-coding loci. Diagnostic options for species identification in practice based on molecular markers, phenotype and MALDI-TOF spectra are presented. A microsatellite typing scheme developed in this study is a powerful tool for the epidemiological surveillance of these emerging pathogens.

KEYWORDS Epizootic fungal infections, Microsatellite typing scheme, Multigene phylogeny, Population genetic structure, Superficial skin infections, Zoophilic dermatophytes

INTRODUCTION

Dermatophytes are a group of fungal pathogens that cause inflammatory and contagious skin diseases that are usuall referred to as dermatophytoses, tinea or ringworm. These are among the most common diseases of warm-blooded animals, including humans, and their prevalence can reach dozens of percent in both human and animal populations (Agnetti et al. 2014; Ahdy et al. 2016; Cafarchia et al. 2010; Duarte et al. 2010; Havlickova et al. 2008; Kupsch et al. 2017; Seebacher et al. 2008). The treatment and prevention of these infections in humans, companion animals and pets require a considerable amount of funding every year (Benedict et al. 2018; Bond 2010; Chermette et al. 2008; Kane and Summerbell 1997; Shenoy and Jayaraman 2019). The incidence of zoonotic dermatomycoses transmitted to humans from livestock decreased significantly in developed countries with the intensification of agriculture, introduction of preventive measures (e.g., vaccination in cattle) and advances in treatment options (Borman et al. 2007; Lund et al. 2014). In contrast, zoonotic infections transmitted from pets remain an important public health concern worldwide (Hubka et al. 2018d). *Microsporum canis* and *Trichophyton mentagrophytes* remain major agents of dermatophytosis in many

domestic animals and cause a significant number of zoonotic dermatophytoses in humans (Hayette and Sacheli 2015). In addition to these well-known causal agents, several emerging zoonotic pathogens are increasingly reported in both humans and pets, and most of them belong to the *Trichophyton benhamiae complex.* The *Trichophyton benhamiae* complex currently comprises six species: *T. benhamiae*, *T. bullosum*, *T. concentricum*, *T. erinacei*, *T. eriotrephon* and *T. verrucosum* (de Hoog et al. 2017; Lysková et al. 2015). These species are predominantly zoophilic, with the exception of anthropophilic *T. concentricum*, an agent of tinea imbricata in tropical regions (Bonifaz et al. 2004; Bonifaz and Vazquez-Gonzalez 2011; Pihet et al. 2008). *Trichophyton verrucosum*, a cause of dermatophytosis in cattle and other ruminants, is one of the best-known members of the complex. It has a worldwide distribution and causes economic losses in the food (negative impacts on milk and meat production), hide and skin industries (Bond 2010; Chermette et al. 2008). The incidence of infections in cattle has decreased in many regions in response to vaccination programmes or changes in agricultural systems, and the rate of infections in humans has decreased proportionally (Seebacher et al. 2008; Lund et al. 2014). By contrast, a lack of prophylaxis accounts for the high infection rates observed in countries such as Italy (Moretti et al. 2013). *Trichophyton verrucosum* grows slowly in culture and frequently produces only chlamydospores as its main microscopic characteristic. In this respect, it is superficially very similar to *T. bullosum*, which causes infections in donkeys and horses, but is much less common and is geographically restricted to the Middle East, Africa and Europe (Lysková et al. 2015; Sabou et al. 2018; Sitterle et al. 2012). Scant data are available on the distribution of *T. eriotrephon,* which is only known from several poorly documented cases of dermatophytosis in humans and dogs (Hubka et al. 2018d; Rezaei-Matehkolaei et al. 2013; Sabou et al. 2018). The remaining two zoophilic species, *T. benhamiae* and *T. erinacei*, are currently considered emerging pathogens, as their incidence as a cause of infections in pets and humans has increased significantly in the last decade (Hubka et al. 2018d). A strikingly high incidence of zoonotic *T. benhamiae* (syn. *Arthroderma benhamiae*) infections, contracted mostly from guinea pigs, is currently reported in various European countries. Although this species was considered less clinically important in recent decades, it became one of the most common agents of zoonotic dermatophytoses after 2010 (Hubka et al. 2018b; Nenoff et al. 2014; Sabou et al. 2018; Symoens et al. 2013; Uhrlaß et al. 2015). It has been shown that the prevalence of the pathogen in guinea pig breeds and pet shops reaches up to 90% (Bartosch et al. 2019; Drouot et al. 2009; Guillot et al. 2018; Kupsch et al. 2017; Overgaauw et al. 2017). Infections occur more frequently in young guinea pigs and are usually asymptomatic. The presence of skin lesions with hair loss (mostly on the muzzle, forehead, ears and around eyes) is also reported in some individuals (Kraemer et al. 2013, 2012). When transmitted to the human host, the infections manifest most commonly as highly inflammatory tinea of glabrous skin and tinea capitis and less commonly as onychomycosis (Nenoff et al. 2014; Skořepová et al. 2014). The presence of asymptomatic infections in animal hosts contributes to the successful spread of the pathogen between animals kept in groups. Such asymptomatic infections also facilitate transmission to pet owners and the occurrence of small familial outbreaks or general infections among pet breeders, pet shop

workers and others. In addition to guinea pigs, this pathogen has been reported in dogs, rabbits, cats, North American porcupines, various small rodents and foxes (Aho 1980; Fréalle et al. 2007; Hiruma et al. 2015; Needle et al. 2019; Sieklucki et al. 2014; Takeda et al. 2012; Ziółkowska et al. 2015). *Trichophyton benhamiae* was originally described as *Arthroderma benhamiae*, a sexual and heterothallic species, from several canine and human infections in North America (Ajello and Cheng 1967). In subsequent years, Takashio (1974, 1977) recognized two races among strains of *T. benhamiae* based on biological compatibility experiments: an "Americano-European" race and an "African" race of *Arthroderma benhamiae*. Furthermore, two phenotypically different groups among strains of the Americano-European race have recently been recognized by different authors and designated the "yellow phenotype" and "white phenotype" strains (Brasch et al. 2016; Hiruma et al. 2015; Nenoff et al. 2014; Symoens et al. 2013). The characterization of mating type genes showed that the MAT1-1-1 idiomorph was significantly prevalent among strains of the yellow phenotype, while MAT1-2-1 prevailed among strains of the white phenotype (Symoens et al. 2013). Similar observations of a lack of one MAT gene or significant bias towards one MAT idiomorph have been made in several other primary pathogenic dermatophytes, while the prevalence of both mating types in a balanced ratio is common in geophilic species (Kosanke et al. 2018; Metin and Heitman 2017).

It was demonstrated that the vast majority of European infections are caused by yellow phenotype strains that emerged relatively recently (Hubka et al. 2014; Nenoff et al. 2014; Symoens et al. 2013; Uhrlaß et al. 2015). The first documented cases of infections due to yellow phenotype strains were recorded between 2002 and 2008 in France and Switzerland (Contet-Audonneau and Leyer 2010; Charlent 2011; Khettar and Contet-Audonneau 2012; Symoens et al. 2013). The first cases in Germany and the Czech Republic were described shortly before 2010, and the pathogen became rapidly epidemic during the following years. Currently, *T. benhamiae* is the most important agent of dermatophytoses transmitted from animals in the Czech Republic and Germany (Hubka et al. 2018b; Hubka et al. 2014; Kupsch et al. 2019; Nenoff et al. 2014; Uhrlaß et al. 2015). The origin of yellow phenotype strains of *T. benhamiae* and the reason for the sudden increase in the incidence of human and animal infections in Europe after 2010 are unknown. As the breeding of guinea pigs has been popular in Europe for decades, the epidemic cannot be explained by a change in pet owner behaviour. Therefore, the spread of a new virulent and highly transmissible genotype/lineage was hypothesized (Čmoková 2015; Hubka et al. 2018d). The occurrence of *T. benhamiae* infections in non-European countries is generally poorly known except for individual reported cases. This is mostly due to insufficient surveillance and a lack of longterm epidemiological studies supported by molecular-based identification of dermatophytes.

In contrast to yellow phenotype strains, white phenotype strains have probably existed worldwide for a long time. Sporadic human and animal infections due to white phenotype strains were described from various European countries, Japan and the USA before the widespread dispersal of yellow phenotype strains in Europe (Aho 1980; Ajello and Cheng 1967; Hejtmánek and Hejtmánková 1989; Kano et al. 1998; Takashio 1974). In Japan, white phenotype strains were first reported in 1996 from an infected

rabbit (Kano et al. 1998); human cases were reported in the following years (Nakamura et al. 2002), and the infections were summarized by Kimura et al. (2015). The increasing number of people breeding pets, together with the increasing import of animals to Japan, is considered a cause of the increased incidence in Japan (Hiruma et al 2015; Kimura et al 2015; Takeda et al 2012). Chronology of reports of white and yellow phenotype strains in various countries is summarized in Fig. 1.

The aim of this study was to elucidate the species boundaries, host spectrum, and population structure of emerging pathogens in the *Trichophyton benhamiae* complex. We examined a large set of clinical isolates associated with human and animal infections that were mostly collected in European countries but also in the USA and Japan. We conducted DNA sequencing of four genetic loci, phylogenetic analyses, and analyses of morphology and physiology to examine whether the previously detected level of phenotypic and genetic variability reflects undescribed species diversity or a high level of infraspecific variability. The levels of recombination/clonality within species and populations, respectively, were estimated by calculating the index of association and determining the ratios between MAT locus idiomorphs. MALDI-TOF MS spectra were compared between species of the *T. benhamiae* complex to test the possibility of their differentiation in the clinical setting. A set of highly variable microsatellite markers were developed to analyse the population structure and relationships between strains with differences in their geographic origin, host spectrum and phenotype. The new taxonomic classification and microsatellite typing scheme proposed in this study will enable the monitoring of changes in the frequencies of individual species and genotypes. This will help to evaluate the results of preventive measures and interventions and is a basic prerequisite for the development of epidemiological studies.

MATERIALS AND METHODS

Source of isolates

More than three hundred strains isolated from human and animal patients with dermatophytosis caused by pathogens from the *T. benhamiae* complex were obtained for this study from various clinical laboratories, hospitals and universities (Table S1): Laboratory for Medical Microbiology (Mölbis, Germany), College of Veterinary Medicine, University of Illinois at Urbana-Champaign (USA), College of Bioresource Sciences, Nihon University (Japan), Laboratory of Mycology, Department of Veterinary Sciences, University of Turin (Italy), and various institutions in the Czech Republic (Institute of Public Health in Ostrava and Usti nad Labem, General University Hospital in Prague, University Hospital in Pilsen, Hospital České Budějovice, Hospital in Pardubice and Labvet veterinary laboratory in Prague). This set of strains was further supplemented with isolates from culture collections, especially BCCM/IHEM Fungi Collection: Human and Animal Health (Brussels, Belgium) and CBS culture collection housed at the Westerdijk Institute (Utrecht, The Netherlands).

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

Molecular studies

DNA was extracted from seven-day-old colonies using the ArchivePure DNA Yeast and Gram2 + Isolation Kit (5 PRIME Inc., Gaithersburg, Maryland) according to the manufacturer's instructions as updated by Hubka et al. (2018c). The quality of the extracted DNA was evaluated by NanoDrop 1000 Spectrophotometer. The ITS rDNA region (ITS1-5.8S-ITS2 cluster) was amplified using the primer set SR6R and LR1 (White et al. 1990) or ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990), partial *gapdh* gene encoding glyceraldehyde-3-phosphate dehydrogenase was amplified with primers GPDF and GPDR (Kawasaki et al. 2011), partial *tubb* gene encoding β-tubulin with primers Bt2a and Bt2b (Glass and Donaldson 1995) and *tef1α* gene encoding translation elongation factor 1-α with primers EFDermF and EF-DermR (Mirhendi et al. 2015). All primer combinations are listed in Table S2. Reaction volume of 20 µL contained 1 µL (50 ng mL^{-1}) of DNA, 0.3 µL of both primers (25 pM mL^{-1}), 0.2 µL of My Taq Polymerase and 4 µL of 5 × My Taq PCR buffer (Bioline, London, UK). PCR conditions followed protocol described by Hubka et al. (2018a). PCR product purification followed protocol of Réblová et al. (2016). Automated sequencing was performed at Macrogen Sequencing Service (Amsterdam, The Netherlands) using both terminal primers. The DNA sequences obtained in this study were deposited into the GenBank database (www.ncbi.nlm.nih.gov) under the accession numbers listed in Table 1.

Phylogenetic analysis

Alignments of the ITS, *gapdh*, *tubb* and *tef1α* regions were performed using the FFT-NS-i option implemented with the MAFFT online service (Katoh et al. 2017). The alignments were trimmed, concatenated and then analysed using maximum likelihood (ML) and Bayesian inference (BI) methods. Suitable partitioning schemes and substitution models (Bayesian information criterion) for the analyses were selected using a greedy strategy implemented in PartitionFinder 2 (Lanfear et al. 2017) with settings allowing introns, exons, codon positions and segments of the ITS region to be independent datasets. The optimal partitioning schemes for each analysed dataset along with basic alignment characteristics are listed in Table S3. The ML trees were constructed with IQ-TREE version 1.4.4 (Nguyen et al. 2015) with nodal support determined by nonparametric bootstrapping (BS) with 1000 replicates. The trees were rooted with *Trichophyton rubrum*. Bayesian posterior probabilities (PP) were calculated using MrBayes 3.2.6 (Ronquist et al. 2012).

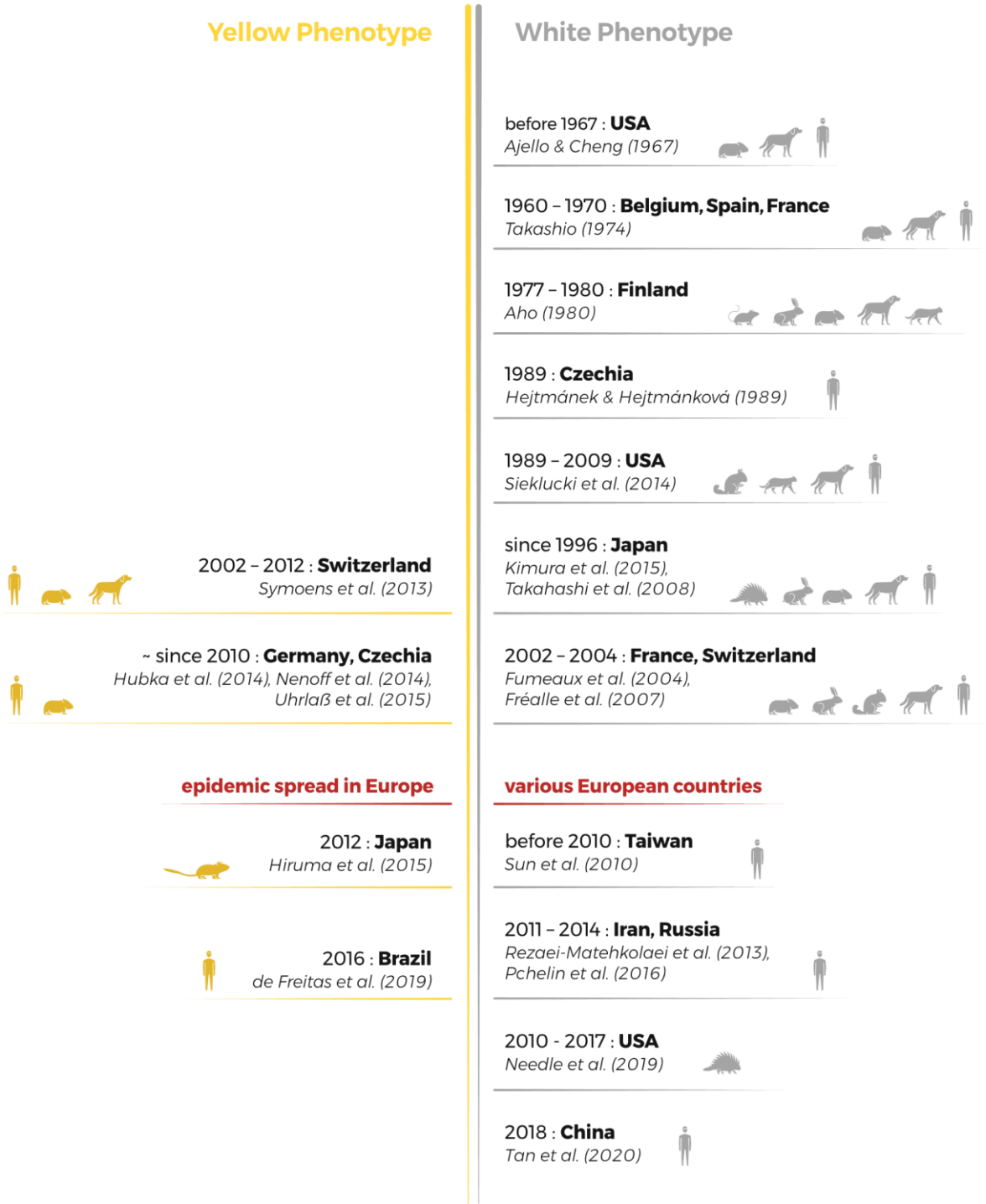


Fig. 1 Chronology of reports of *Trichophyton benhamiae* phenotypes from various countries. Yellow-phenotype isolates correspond to *T. benhamiae* var. *luteum* proposed in this study. White-phenotype strains correspond to *T. benhamiae* var. *benhamiae* and two novel species proposed here: *T. europaeum*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

Optimal partitioning scheme and substitution models were selected as described above and are listed in Table S3. The analysis ran for 1 0^7 generations, two parallel runs with four chains each were used, every 1 000^{th} tree was retained, and the first 25% of trees were discarded as burn-in. The convergence of the runs and effective sample sizes were checked in Tracer v1.6 (https ://tree.bio.ed.ac.uk/softw are/trace r). The modified complex indel coding (MCIC) algorithm implemented in SeqState version 1.25 (Müller 2005) was used to code gaps. The TCS network method (Clement et al. 2000) was used to generate haplotype networks implemented in the program PopART (Leigh and Bryant 2015).

Development of microsatellite markers

Microsatellite motifs were identified in the available genomic sequence of *T. europaeum* CBS 112371 = IHEM 20161 = CCF 6479 (https ://www.broad insti tute.org/) using WebSat online software (Martins et al. 2009). The same program suggested optimal primers for the amplification of target loci. We selected di-, tri-, and tetranucleotide repeats based on the loci with the highest repeat numbers. Interrupted repeats as well as loci containing two or more repeat motifs within the fragments delimited by particular primer pairs were excluded. A pilot set of eight strains was used to evaluate microsatellite polymorphism for all candidate loci following the method of Schuelke (2000). PCR conditions were as follows: one cycle 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 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 loci exhibiting the highest level of polymorphism was selected from the 160 tested loci. The PCR products were screened for the presence of undesirable polymorphisms in the microsatellite flanking regions and the presence of polymorphisms in the microsatellite regions by sequencing. Emphasis was also placed on the selection of loci that were approximately uniformly distributed across the genome. Primer–primer interactions were checked before assembling multiplexes using Multiple Primer Analyzer (https ://www.therm oscie ntific bio.com/webtools/multip lep rimer /). The forward primers of ten selected loci were tagged with fluorescent dye and arranged into a single multiplex panel (Table 2). The reaction volume of 5 µL for PCR contained 50 ng DNA, 0.5 µL of the mixture of primers and 2.5 µL of Multiplex PCR Master Mix (QIAGEN, Germany). The PCR conditions were chosen according to the manufacturer's recommendations. The PCR products (diluted in water 1:50) were mixed with 10 µL of deionized formamide and 0.2 µL of the GeneScan™ 600 LIZ size standard and denatured for 5 min at 95 °C, followed by analysis on an ABI 3100 Avant Genetic Analyzer.

Statistical analysis of microsatellite data

The discriminatory power of these newly designed loci was calculated using Simpson's index of diversity as described previously (Hunter and Gaston 1988). A binary and allele data matrix was created using GeneMarker 1.51 software (SoftGenetics, LLC, State College, PA, USA) and used to estimate the similarities between individuals using Jaccard's similarity coefficient calculation in the program FAMD

(Schlueter and Harris 2006). A neighbour-joining tree based on Jaccard's similarity coefficient matrix was constructed using the same software. Genetic distances were calculated from the same matrix and used for the construction of the NeighborNet network in the SplitsTree 4 program (Huson 1998).

A Bayesian model-based clustering algorithm with a clustering number (K) = 1–10 was applied to the clonecorrected allele data matrix using the software STRU CTU RE (Pritchard et al. 2000). Ten simulations were calculated at the www.biopo rtal.uio.no server (Lifeportal, University of Oslo) using the admixture model and 1 0^6 MCMC replicates; 5×10^8 replicates were discarded as burn-in. The noadmixture model and uncorrelated allele frequencies were chosen for the analysis. The optimal clustering number (K) was estimated using ΔK and similarity coefficients (Evanno et al. 2005), and both values were calculated using the script structure-sum (Ehrich 2006) in the R version 3.3.4 program (R Core Team 2016).

The genetic variability within and between clusters was analysed for ten variable loci on the clone-corrected dataset via analysis of molecular variance (AMOVA) (Excoffier et al. 1992) in the Arlequin program (Schneider et al. 2000). The degree of gene flow among clusters was estimated using a pairwise fixation index (F_{ST}) and a coefficient of genetic differentiation (G_{ST}) calculated in Arlequin (Schneider et al. 2000) and POPGENE (Yeh et al. 1999), respectively.

The degree of clonality or recombination within particular clusters was estimated by calculating the index of association (I_A) in the program MultiLocus 1.3 (Agapow and Burt 2001), which is used for measuring the linkage disequilibrium between alleles and is useful in inferring the occurrence of cryptic recombination in putatively asexual populations (Burt et al. 1996). Random mating is suggested if no linkage is detected between the alleles of different loci (randomly distributed alleles); in that case I_A is expected to be nearly zero or zero. We tested for significant deviation from 10,000 random multilocus permutations of genotypes under a random mating model.

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

MAT locus determination

A partial sequence of the MAT1-1-1 gene encoding the alpha box domain was amplified with the primers MF1 and MF5, and a partial sequence of the MAT1-2-1 gene encoding the high mobility group (HMG)

domain was amplified with the primers Ab_HMG_F and Ab_HMG_R or TmHMG3S and TmHMG3R (Kano et al. 2012; Kosanke et al. 2018; Symoens et al. 2013). The PCR volume of 10 µL contained 25 ng of DNA, 0.15 µL of both primers (25 pM mL^{-1}), 0.15 µL of My Taq Polymerase and 2 µL of buffer. The PCR conditions are described above. The PCR products were visualized in an electrophoretogram (1% agarose gel with 0.5 µg mL^{-1} ethidium bromide). Several PCR products of each MAT idiomorph were subjected to sequencing for the confirmation of product specificity.

Phenotypic studies

The morphology of the colonies on malt extract agar (MEA, HiMedia, Mumbai, India) at 25 °C was documented in all strains. At least five strains from each species (if available) were subjected to a detailed analysis that involved macromorphology on MEA, potato dextrose agar (PDA, Himedia, Mumbai, India) and Sabouraud dextrose agar [SAB, Atlas (2010)] at 25, 30 and 37 °C. The macromorphology of the colonies was documented using an Olympus SZ61 or Canon EOS 500D binocular loupe (with Olympus Camedia C-5050 Zoom camera). Colony colour determinations were made using the ISCC-NBS Centroid Colour Charts (Kelly 1964); https: //tx4.us/nbs/nbs-1.htm.
Micromorphology was documented using an Olympus BX-51 microscope. Particular micromorphological characteristics were recorded at least 35 times for each isolate (at least five strains selected per species). The variance inflation factor (VIF) was assessed before performing the analysis of variance to test the correlation between variables. Statistical differences in particular phenotypic characteristics were tested with one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test in program R version 3.3.4 (R Core Team 2016).

MALDI-TOF mass spectrometry

The cultivation of strains from the *T. benhamiae* clade (up to five strains from each species, if available) was performed in liquid cultivation medium for 22–24 h. The strains were prepared according to Schrödl et al. (2012) and analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). In brief, for MALDITOF MS analysis, all samples were prepared using the liquid cultivation method and ethanol / formic acid extraction method. One milliliter of each overnight culture was centrifuged for 2 min at about 10,000 × *g*. The supernatant was carefully removed and the fungal pellet was resuspended in 1 ml water, mixed thoroughly, and centrifuged for further 5 min at 10,000 × *g*. After removing the supernatant the pellet was resuspended in a mixture of 300 µL bidistilled water and 900 µL absolute ethanol. After centrifugation, the fungal cells were dried shortly and mixed thoroughly with 50 µL of 70% formic acid and 50 µL pure acetonitrile, followed by centrifugation for 2 min at 10,000×*g*. A volume of 1 µL supernatant was placed onto a MALDI target plate (Bruker Daltonik GmbH, Germany) and allowed to dry at room temperature. Eight MALDI target positions per strain were prepared in parallel. Each sample position (including one Bruker Bacterial

Table 1 Shortened list of *Trichophyton* strains used for phylogeny reconstruction and sequence accession numbers

Clade/species	Culture collection numbers[a,b]	GenBank/ENA/DDBJ accession numbers[c]			
		ITS	*gapdh*	*tef1α*	*tubb*
Trichophyton benhamiae clade		**LR794129**	**LR794235**	**LR794260**	**LR794285**
Trichophyton benhamiae var. *benhamiae*	IHEM 4710 = CBS 623.66 = ATCC 16781 = CCF 6484 = IMI 124768 = CDC X-797 = CECT 2892 = IP 1064.74 = NCPF 0410 = RV 23303 = UAMH 2822 = TM-20 ^{T} & 16 other isolates with identical MLST genotype (Table S1)				
	USA 3355 & USA 3360 = CCF 6486 with identical MLST genotype	**LR794130**	**LR794236**	**LR794261**	**LR794286**
Trichophyton benhamiae var. *luteum*	IHEM 25068 = CCF 6500 ^{T} & 235 other isolates with identical MLST genotype (Table S1)	**LR794131**	**LR794237**	**LR794262**	**LR794287**
Trichophyton concentricum	CBS 196.26 = IFO 5972 ^{T}	**LR794126**	**LR794232**	**LR794257**	**LR794282**
	IHEM 5470 = CCF 5302	**LR794127**	**LR794233**	**LR794258**	**LR794283**
	IHEM 13435 = CCF 5303 = RV 30442	**LR794128**	**LR794234**	**LR794259**	**LR794284**
Trichophyton europaeum	IHEM 22725 = CCF 6499 ^{T} & 38 other isolates with identical MLST genotype (Table S1)	**LR794134**	**LR794240**	**LR794265**	**LR794290**
	IHEM 25139 = CBS 806.72 = RV 14387 = CCF 6480 = ATCC 28061 = IFM 54422	**LR794135**	**LR794241**	**LR794266**	**LR794291**
Trichophyton japonicum	IHEM 17701 = ATCC 28063 = CBS 807.72 = CCF 6481 = CECT 2894 = RV 14988 ^{T} & 17 other isolates with identical MLST genotype (Table S1)	**LR794132**	**LR794238**	**LR794263**	**LR794288**
	DMF 1658 & DMF 3061 with identical MLST genotype	**LR794133**	**LR794239**	**LR794264**	**LR794289**
Trichophyton erinacei clade					
Trichophyton erinacei	CBS 511.73 = ATCC 28443 = IMI 101051 = NCPF 375 ^{T}	**LR794136**	**LR794242**	**LR794267**	**LR794292**
	CBS 344.79	**LR794137**	**LR794243**	**LR794268**	**LR794293**
	IHEM 19619 = RV 28925	**LR794138**	**LR794244**	**LR794269**	**LR794294**
	IHEM 19621 = RV 28927	**LR794139**	**LR794245**	**LR794270**	**LR794295**
	ATCC 22442 = CBS 474.76 = IMI 117369 = NCPF 504	KJ606082	–	–	KJ606178
	ATCC 28446	MN737936	EF631657	–	EF631720
Trichophyton eriotrephon	CBS 220.25 ^{T}	FM992674	**LR794246**	**LR794271**	**LR794296**
	IHEM 24340	MK298922	**LR794247**	**LR794272**	**LR794297**
	isolate 361	JN134089	–	KM678176	–
	isolate 363	JN134090	–	KM678177	–
Trichophyton verrucosum	CBS 365.53 ^{T}	**LR890161**	**LR890162**	**LR890163**	**LR890164**

[a] *ATCC* American Type Culture Collection, Manassas, USA; *IHEM (BCCM/IHEM Fungi Collection: Human and Animal Health)* Belgian Coordinated Collections of Micro-organisms, Fungi Collection: Human and Animal Health, Sciensano, Brussels, Belgium, *CBS* Centraalbureau voor Schimmelcultures, Utrecht, Netherlands, *CCF* Culture Collection of Fungi, Prague, Czech Republic, *CCM*

Table 1 (continued)

Clade/species	Culture collection numbers[a,b]	GenBank/ENA/DDBJ accession numbers[c]			
		ITS	*gapdh*	*tef1α*	*tubb*
	CCF 4612	LN614529	**LR794248**	**LR794273**	**LR794298**
	CCF 4613	LN614530	**LR794249**	**LR794274**	**LR794299**
	CCF 4889	LN614527	**LR794250**	**LR794275**	**LR794300**
Trichophyton bullosum clade *Trichophyton africanum*	IHEM 4032 = ATCC 28064 = CCF 6493 = RV 25293 = CM 3440 [T]	**LR794140**	**LR794251**	**LR794276**	**LR794301**
	IHEM 19628 = RV 40614	**LR794142**	**LR794253**	**LR794278**	**LR794303**
	IHEM 4033 = ATCC 28065 = CBS 808.72 = CECT 2895 = NCPF 456 = RV 27926	**LR794141**	**LR794252**	**LR794277**	**LR794302**
Trichophyton bullosum	CBS 363.35 = LP 770 [T]	**LR794143**	**LR794254**	**LR794279**	KT155552
	CCF 4831	**LR794144**	**LR794255**	**LR794280**	**LR794304**
	CCF 5730	**LR794145**	**LR794256**	**LR794281**	**LR794305**
	CBS 557.50	FM992676	–	–	KT155587
Outgroup					
Trichophyton rubrum	CBS 202.88	AOKX01000074	AOKX01000074	AOKX01000074	AOKX01000074

(F-) Czech Collection of Microorganisms, Brno, Czech Republic, *CDC* Centers for Disease Control, Atlanta, USA, *CECT* Spanish Type Culture Collection, Valencia, Spain, *DSM* Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, *FMR* Faculty of Medicine, Reus, Spain, *IMI* CABI's collection of fungi and bacteria, Egham, UK, *IP* Institut Pasteur Culture Collection, Paris, France, *NCPF* National Collection of Pathogenic Fungi, London, UK, *KMU* Kanazawa Medical University, Ishikawa, Japan, *NHL* National Institute of Hygienic Sciences, Tokyo, Japan, *NRRL* Agricultural Research Service Culture Collection, Peoria, Illinois, USA, *RV* former collection of Raymond Vanbreuseghem (now incorporated in BCCM/IHEM), *UAMH* UAMH Centre for Global Microfungal Biodiversity, University of Toronto, Toronto, Canada [b] Ex-type strains are designated by a superscript "T" [c] Accession numbers in bold were generated in this study

Table 2 Microsatellite markers developed for genotyping of *Trichophyton benhamiae* clade members

Foward primer	Sequence (5′–3′)	Reverse primer	Sequence (5′–3′)	Final concetration (μM)[a]	5′ fluoresent dye	Fragment size
$(CT)_{21}$	GTG ATG TAT GTA TGT CCC CGTG	$(CT)_{21}$_R	AAG AGA GAG CGA GAG TGG AAGA	0.1	NED	228–258
$(TAG)_{16}$	TGT TTG GAT GCT GAT GTT AAGG	$(TAG)_{16}$_R	TTC TTC GTC TTC TCC TGT TTCC	0.1	VIC	205–302
$(TC)_{17b}$	CAA TCA GGC GTT TAT CCT CTCT	$(TC)_{17b}$_R	GGA TCA CAC TAA AGC TGG CAAT	0.1	VIC	328–360
$(TCA)_{16}$	AAA CGA CTT CTT TCG ATA CCCA	$(TCA)_{16}$_R	CTT CTT GCT TCT TCG GGT TAAG	0.1	PET	268–316
$(TC)_{17a}$	GGT AGA CAC TCG ACA ACA CACG	$(TC)_{17a}$_R	GAG GAT AGC ACG GAA CAA AGAC	0.1	FAM	265–300
$(AG)_{18}$	GCG TTA ATT TCT CAC CGT TACC	$(AG)_{18}$_R	CGT CGT CTC TCT TGT GTT TGAC	0.25	FAM	367–377
$(CT)_{21b}$	CTT CTT GTT GCT GCT CTT GTTG	$(CT)_{21b}$_R	GAC GTA TCC TAG ACA TCC TCCG	0.1	NED	262–295
$(TC)_{19}$	GCC CGC TTA TTG AGT CAG TC	$(TC)_{19}$_R	ACC TGA CTC TCG CCA TCT GT	0.1	FAM	227–249
$(TC)_{20}$	TCT TTT CGT CTC CTT CTT CCTG	$(TC)_{20}$_R	TCT GTG TTC TTT TCT GAC GCTG	0.1	PET	183–197
$(AG)_{21}$	CTG AGC CCA TAT CCA AAT TCTC	$(AG)_{21}$_R	CTG AGT TAA GGA GGC AAT TCCA	0.25	PET	325–369

[a] Optimal concetration for multiplex PCR reaction.

Test Standard position) was overlaid with 1 μL of matrix (HCCA portioned; Bruker Daltonik GmbH, Germany) and air dried at room temperature. MALDI-TOF MS measurement was conducted on a Microflex LT benchtop instrument operated by FlexControl software (Bruker Daltonik GmbH, Leipzig, Germany). Spectra were acquired in linear positive mode at a laser frequency of 200 Hz within a mass range from 2000 to 20,000 Da by using the standard flexControl and AutoX methods. For each sampled spot up to three sum spectra were accumulated resulting in 24 MALDI spectra per strain. Finally, five spectra were selected for better spectra handling and visualization.

RESULTS

Phylogeny of the *Trichophyton benhamiae* complex

We assessed 340 combined ITS, *gapdh*, *tubb* and *tef1-α* sequences from members of the *T. benhamiae* species complex (TBSC) in the phylogenetic analysis. The final alignment included 2371 characters, with 247 variable and 152 parsimony informative sites, and *Trichophyton rubrum* CBS 202.88 was used as the outgroup. The detailed alignment characteristics together with the partitioning schemes and substitution models are listed in Table S3. The isolation source and accession numbers for the DNA sequences are available in Table 1 and Table S1. The alignments are available in the online supplementary material.

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

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

Species from the *T. benhamiae* clade show a low level of intraspecific genetic variability. In total, there are only seven unique multilocus genotypes (MLST) among 318 isolates belonging to the *T. benhamiae* clade (Fig. 3). Two MLST genotypes are present among *T. benhamiae* strains, represented by a single substitution in the *tef1-α* gene (Fig. S4). Two MLST genotypes are present in *T. japonicum*, caused by a single substitution in the ITS1 region. *Trichophyton japonicum* can be differentiated from the closely related *T. europaeum* by a single substitution in the ITS region and four conserved substitutions in the *gapdh* gene (Figs. S2-S5). No intraspecific variability is detectable among the isolates of *T. europaeum*. The only exception is the isolate of "*T. europaeum*" IHEM 25139, which presents an abnormal ITS1

region sequence that contains 6 additional substitutions compared to the *T. europaeum* isolates. Some of these positions are critical for the differentiation of the *T. europaeum*/*T. japonicum* lineage from *T. benhamiae* s. str., suggesting that this strain could be a hybrid between *T. benhamiae* clade species. The *gapdh* gene sequence of IHEM 25139 is typical of *T. europaeum*.

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

The *T. erinacei* clade comprises three species: *T. erinacei*, an agent of mycoses in hedgehogs (genera *Erinaceus*, *Aterelix*); *T. verrucosum*, an agent of cattle ringworm; and *T. eriotrephon*, with poorly known ecological characteristics (Fig. 2). All analyzed isolates of *T. erinacei* and *T. verrucosum* presented the MAT1-2-1 idiomorph, while *T. eriotrephon* exhibited only the MAT1-1-1 idiomorph. The *T. bullosum* clade contains three isolates of the African race of *Arthroderma benhamiae* from humans, and *T. bullosum* which is a causal agent of dermatomycoses in horses and donkeys. Isolates of the African race apparently represent an independent taxonomic entity, and we propose the name *T. africanum* for this species (Fig. 2). Both MAT gene idiomorphs were detected in *T. africanum*, while *T. bullosum* isolates exhibited only the MAT1-1-1 idiomorph.

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

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

This newly developed microsatellite typing scheme was applied to a total number of 318 isolates belonging to the *T. benhamiae* clade. Forward primers of all loci were marked with fluorescent dye and arranged in a multiplex panel (Table 2). The highest number of alleles was found at the TAG16 (n = 12) locus, followed by the CT21 (n = 10) locus. In contrast, the fewest alleles were found in the AG21 (n = 5) and TC20 (n = 5) loci. The remaining loci included 6–9 alleles (Table S4). All loci were successfully amplified in all examined strains (null alleles were not found). The dependence of genotypic diversity on the number of loci showed that a sufficient number of markers was used to resolve the population structure of the *T. benhamiae* clade. It was apparent from the curves (Fig. 4) that genetic diversity would not increase significantly with the addition of additional markers.

A Bayesian model-based clustering algorithm was used to determine how many groups were included in the dataset. The highest ΔK value was observed at K = 6, and a much lower peak was present at K = 4 (Fig. 5). The estimated population structure inferred from this analysis is shown in Fig. 5. The analysis revealed a total of 41 genotypes among *T. benhamiae* clade isolates clustering into six clusters (C1–C6).

The distribution of the isolates into clusters was correlated with their geographic distribution and main primary hosts (Fig. 6). The cluster C1 was found most abundantly in Europe and was associated with guinea pigs. These isolates are responsible for the current outbreak of infections in Central Europe and consist exclusively of yellow phenotype strains. We propose the name *T. benhamiae* var. *luteum* for this cluster. Clusters C2 and C3 comprised white phenotype strains from North America isolated mostly from dogs and characterized by highly variable microsatellite data (*T. benhamiae* var. *benhamiae*). Cluster C4 (*T. japonicum*) comprised the majority of strains from Japan analysed in this study and some European strains (rabbits, guinea pigs and human infections contracted from them). Cluster C5 (*T. europaeum*) comprised strains from Europe (infections mostly contracted from guinea pigs). The isolate IHEM 25139 was assigned to *T. europaeum* but its haplotype was intermediate between *T. europaeum* and *T. japonicum* (alleles CT21 and CT21b were characteristic of *T. japonicum,* while the remaining 8 alleles were from *T. europaeum*). Cluster C6 was represented by three human isolates of *T. concentricum* from tropical regions.

The clustering based on the microsatellite data was correlated with MAT gene distribution and single-gene DNA data (*tubb* gene was excluded due lack of variability in *T. benhamiae* clade) (Fig. 7, Fig. S6). It is evident from the visualisation that clustering of isolates according to the single-gene genotype and MAT idiomorphs was in general agreement with microsatellite data and proposed by Bayesian clustering (the peak of ΔK was observed at K = 6); clones were discarded from the analysis; the number of isolates representing each haplotype is indicated in parentheses following the isolate number; the species hypothesis. However, the clusters C1–C3 are not supported by any DNA locus sequences in study and are only distinguishable by microsatellites. *Trichophyton benhamiae* var. *luteum* (C1) was characterized by low variability of microsatellite data and exclusively consisted of isolates with MAT1- 1-1 idiomorph. The isolates of *T. benhamiae* var. *benhamiae* cluster C2 were exclusively of the MAT1-2-1 idiomorph, while those of cluster C3 were exclusively of the MAT1-1-1 idiomorph. Despite obvious phenotypic and population genetic differences between *T. benhamiae* var. *benhamiae* and *T. benhamiae* var. *luteum*, these two varieties are not distinguishable by any of the DNA sequence markers used in this study. The only detected DNA sequence variant, represented by a single substitution in the *tef1-α* gene, did not fully correspond to the two varieties delimited by microsatellite markers (Fig. 7).

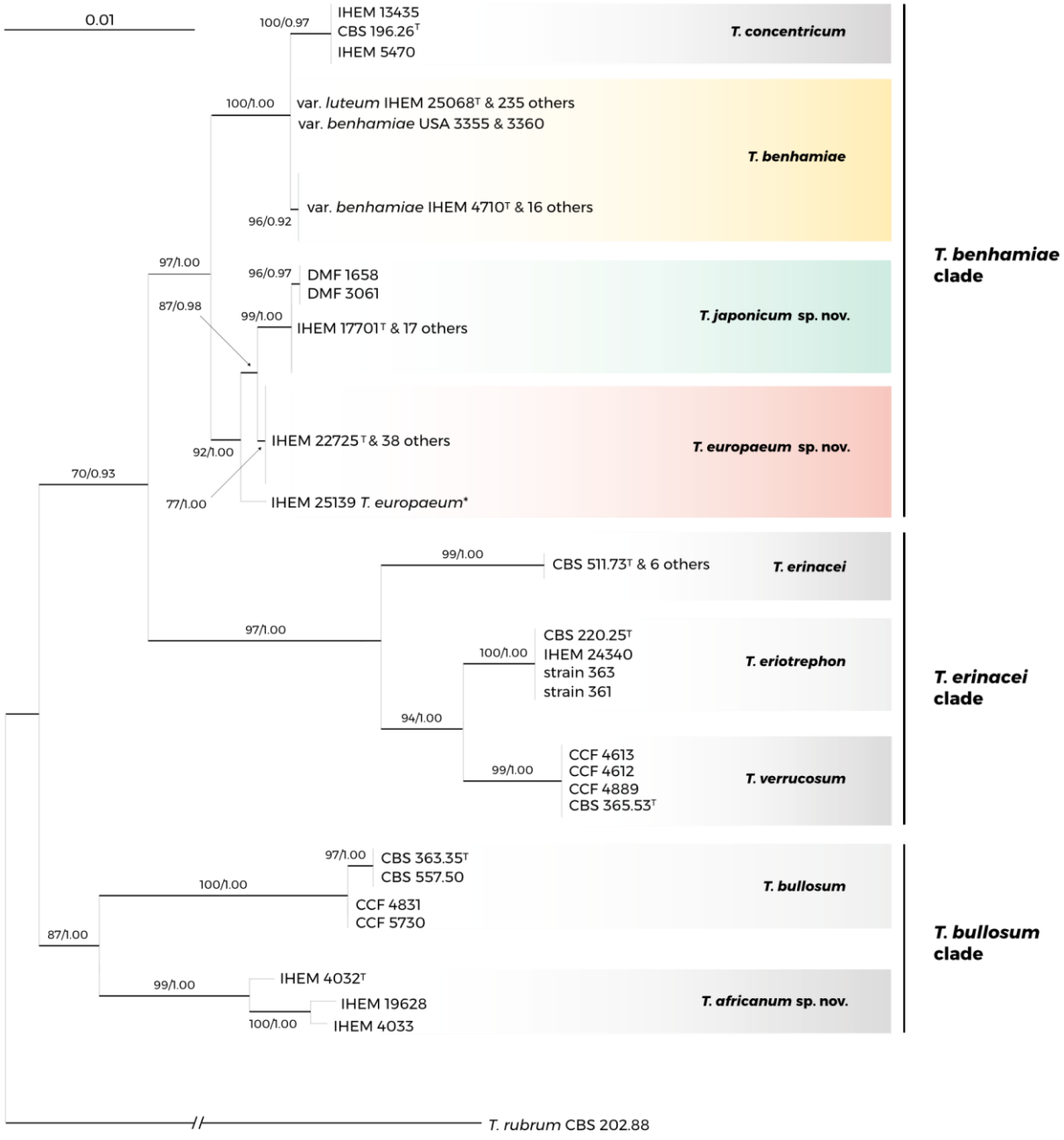


Fig. 2 Multilocus phylogeny of the *Trichophyton benhamiae* complex inferred with the maximum likelihood method based on the gapdh, tubb, ITS rDNA and tef1-α loci (alignment characteristics, partitioning scheme and substitution models are listed in Table S3). Maximum likelihood bootstrap values and Bayesian posterior probabilities are appended to the nodes; only support values higher than 70% and0.90, respectively, are shown. The ex-type strains are designated with a superscripted *T.* *Trichophyton rubrum* CBS 202.88 was used as the outgroup

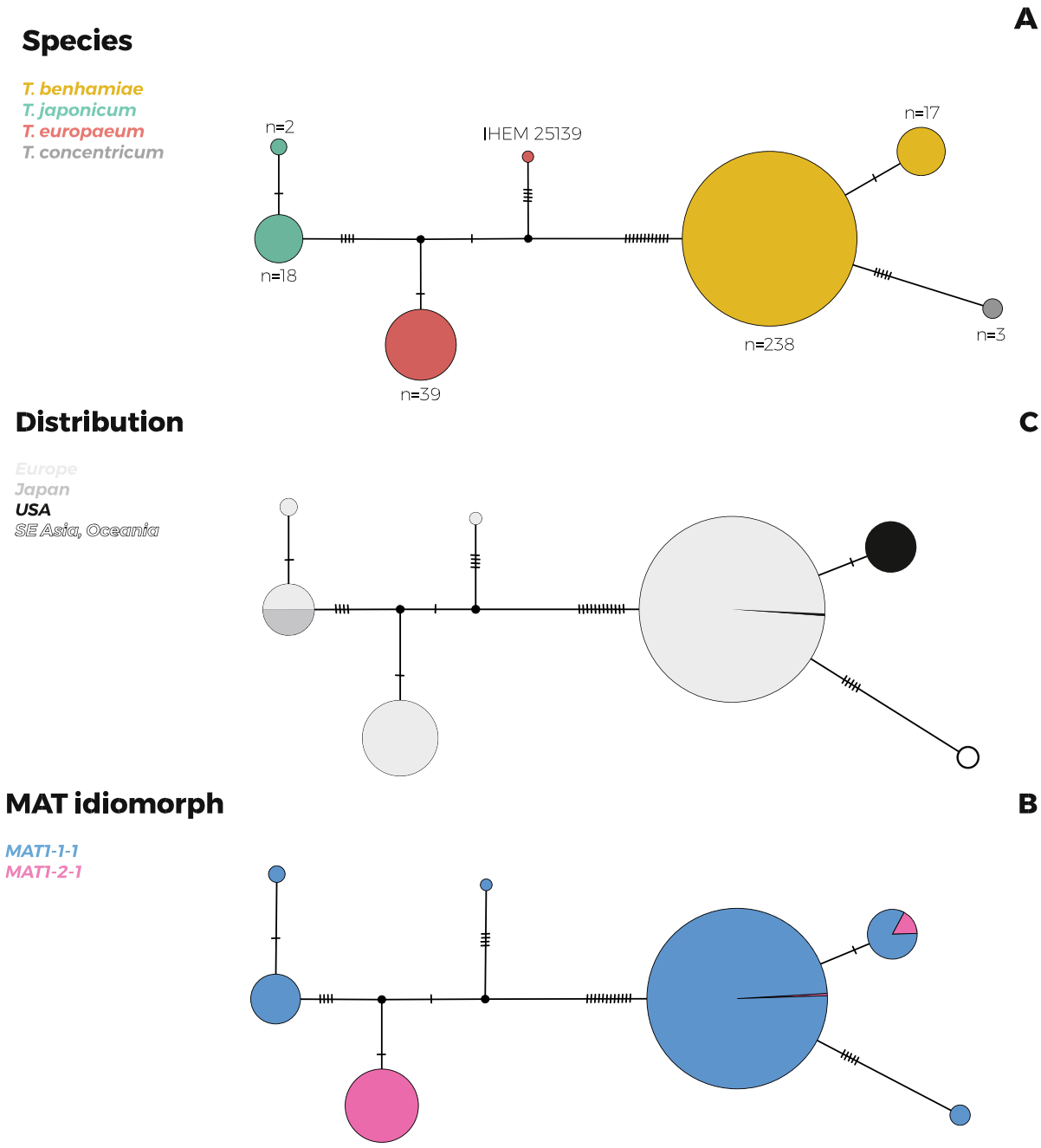


Fig. 3 Haplotype network of the *Trichophyton benhamiae* clade based stitutions (indels are excluded). The upper figure shows the species on multilocus data (*gapdh*, *tubb*, ITS rDNA and *tef1-α* loci). Haplo- identity and genotypic diversity, the middle figure shows the distributypes are indicated by circles whose sizes correspond to the number tion of MAT gene idiomorphs, and the lower figure shows the geoof analysed strains, and dashes on the connecting lines indicate sub- graphic distribution of particular genotypes

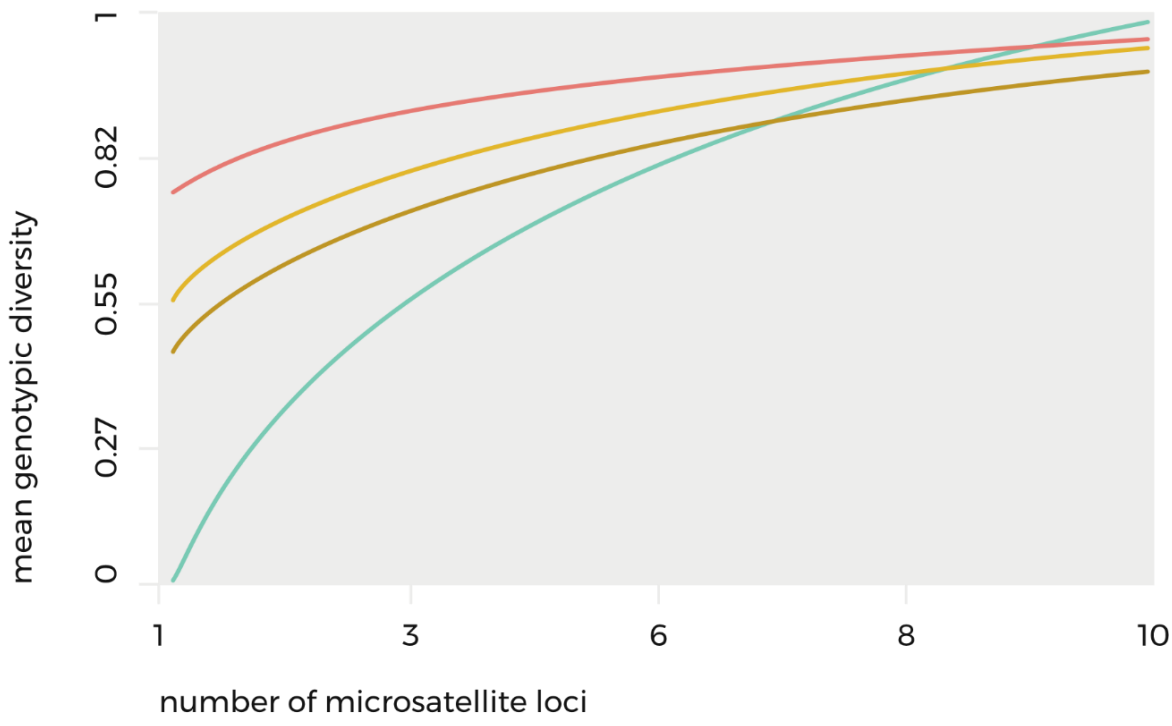


Fig. 4 Plot of mean genotypic diversity as a function of the number of microsatellite loci

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

Population characteristics were calculated from microsatellite data to test significance of clonal expansion versus recombination, and genetic diversity within clusters. Besides the inability to reproduce sexually due to missing opposite mating type in most of species, the clonality is indicated by the skewed distribution of pairwise differences between individuals (Fig. 8). Consequently, all populations are genetically uniform which is evident from low value of Nei's gene diversity (D) (Table S5) that ranged from 0.02 in *T. benhamiae* var. *benhamiae* cluster 3 to 0.156 in *T. benhamiae* var. *benhamiae* cluster 2. The low Nei's genotype diversity (Dg = 0.35) of *T. benhamiae* var. *luteum* compared to other taxa reflects the fact that the population consisted of several abundant clones (Table S5). Asexual reproduction prevails in all populations for long time which is supported by the low effective number of genotype (G_{eff}) values that were significantly lower than observed number of genotypes (Table S5). The exception was *T. benhamiae* var. *benhamie* cluster C2 (Table S5). However,

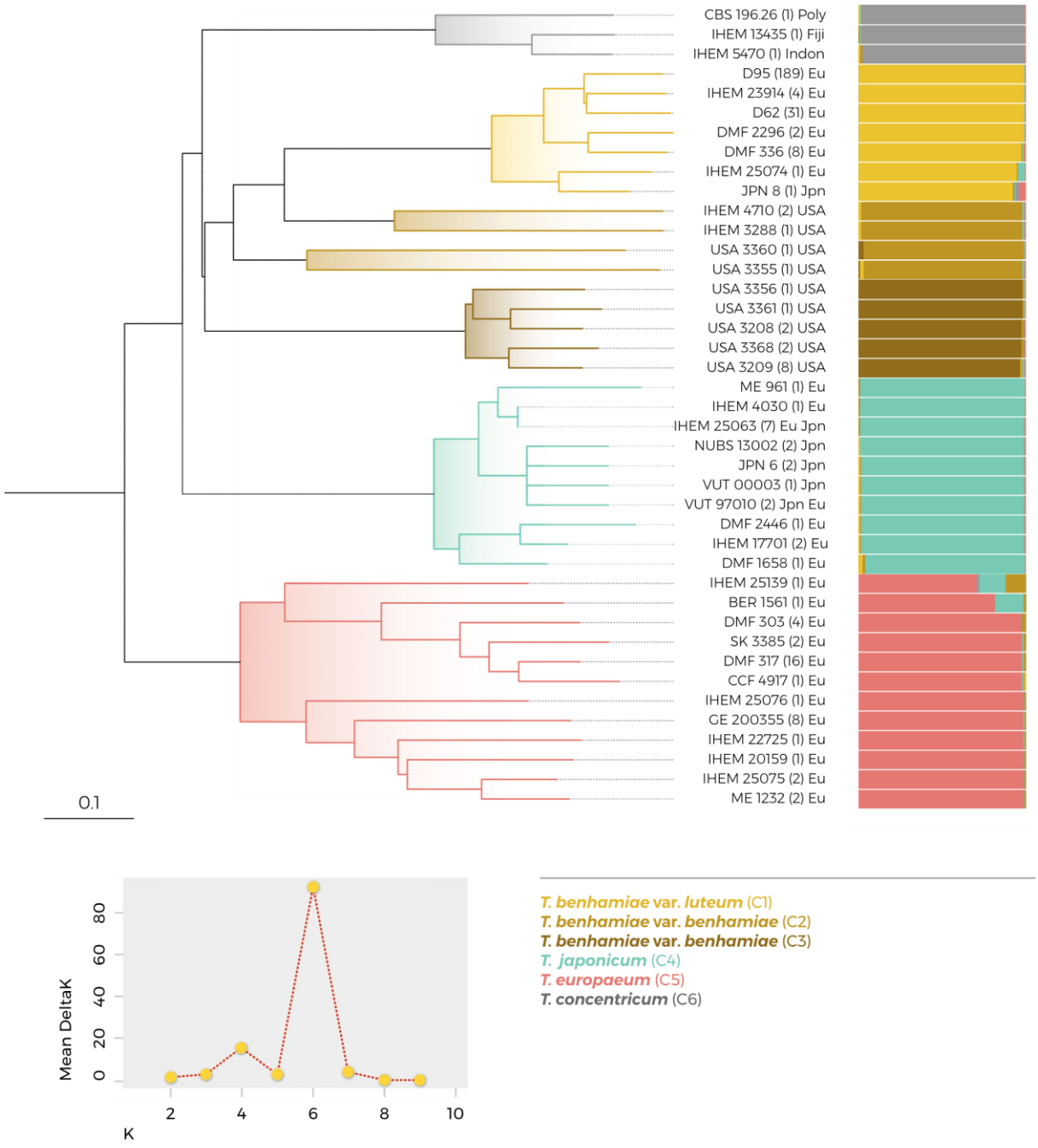


Fig. 5 The population structure of the *Trichophyton benhamiae* clade (ten microsatellite loci, 318 isolates). The neighbour-joining tree was calculated from the multilocus microsatellite profiles using the Jaccard distance matrix measure in FAMD 1.3 (Schlueter and Harris 2006) and is used solely for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

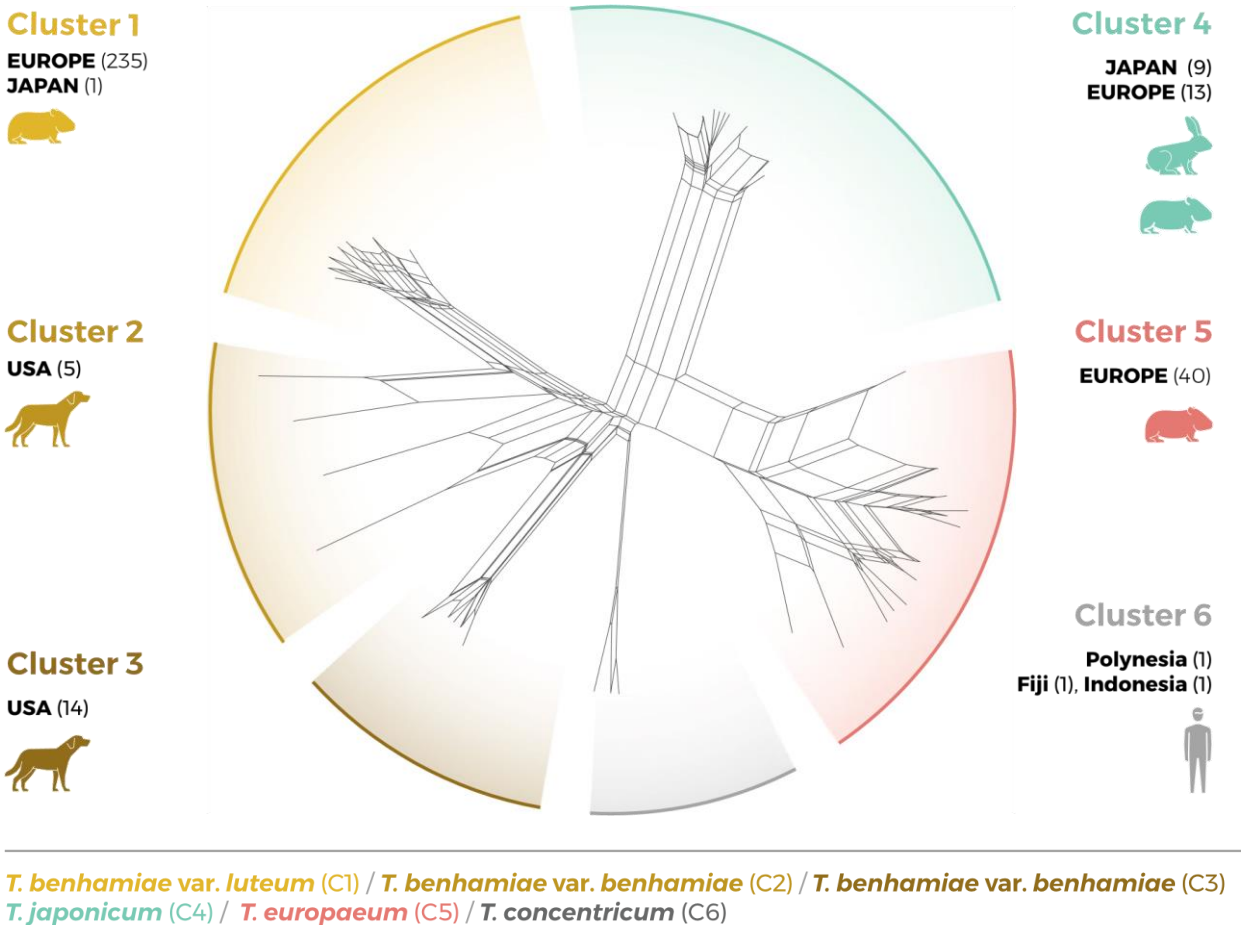


Fig. 6 Population structure of the *Trichophyton benhamiae* clade revealed by the analysis of ten microsatellite loci in 318 strains. The NeighborNet network was built with FAMD 1.3 software and visualized in SplitsTree 4.13 using the Jaccard index-based distance matrix (Delta score: 0.1778, Q-residual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

recombination in cluster C2 was not confirmed by calculation of index of association (I_A) (Table S5), possibly due to low number of samples available. The recombination was not rejected only in *T. europaeum* population according to I_A on significance level $p < 0.05$ ($I_A = 0.24$, $p < 0.0042$) (Fig. 9, Table S5).

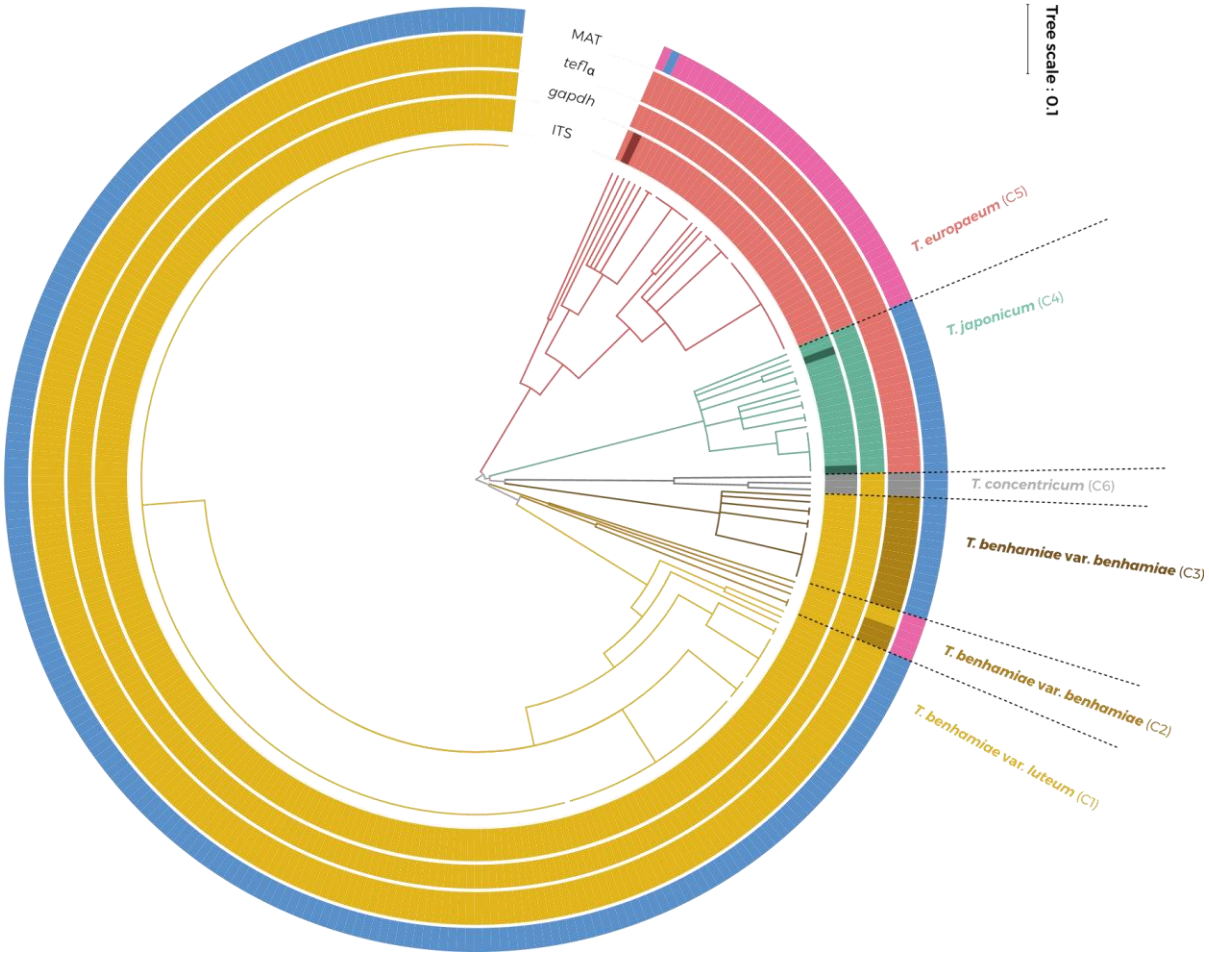


Fig. 7 Phylogenetic tree of the *Trichophyton benhamiae* clade revealed by the analysis of ten microsatellite loci in 318 strains constructed in FAMD software using a Jaccard index-based distance matrix. Coloured circles display the genotype diversity of the ITS, *gapdh* and *tef1-α* loci and the distribution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

To test cluster-specific differences, AMOVA was performed on the microsatellite data. The diversity between six clusters contributed to a total variability of 68.1%, while the diversity within clusters contributed to only 31.9% ($p < 0.0001$). Thus, there is a low level of genetic information exchange between clusters, reflected in a high number of fixed alleles ($F_{ST} = 0.89$, $G_{ST} = 0.75$, $p < 0.0001$). *Trichophyton concentricum* and *T. benhamiae* var. *benhamiae* cluster C2 shared the greatest number of alleles in common ($F_{ST} = 0.451$, $G_{ST} = 0.46$). The lowest number of shared alleles was found between *T. benhamiae* var. *luteum* and all other clusters ($F_{ST} = 0.90–0.95$; Table S6).

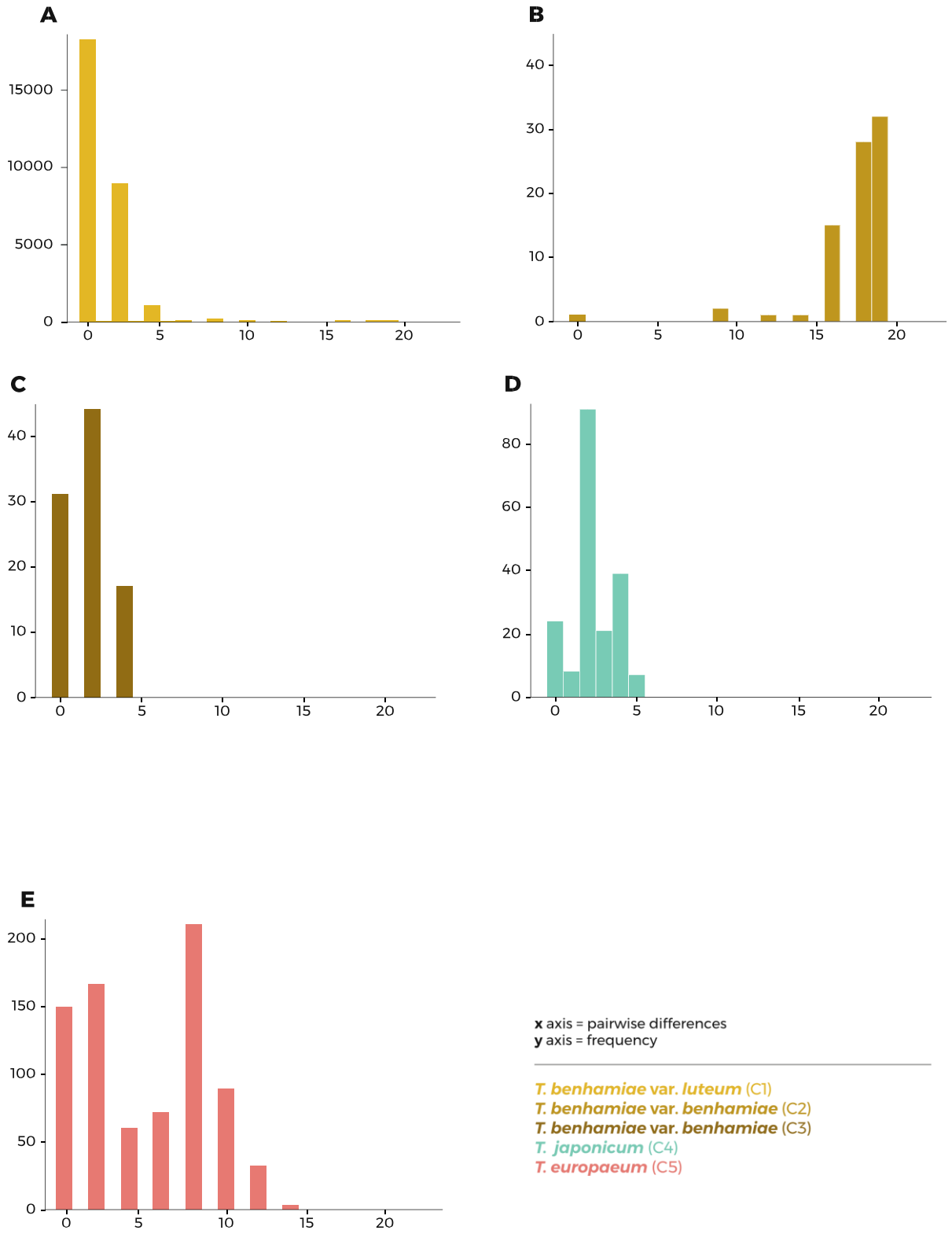


Fig. 8 Histograms showing the frequency of pairwise genetic differences among individuals within species/populations: *Trichophyton Trichophyton europaeum* (**e**) *benhamiae* var. *luteum* (**a**); *Trichophyton benhamiae* var. *benhamiae* clusters C2 (b) and cluster C3 (c); *Trichophyton japonicum* (d); *Trichophyton europaeum* (e).

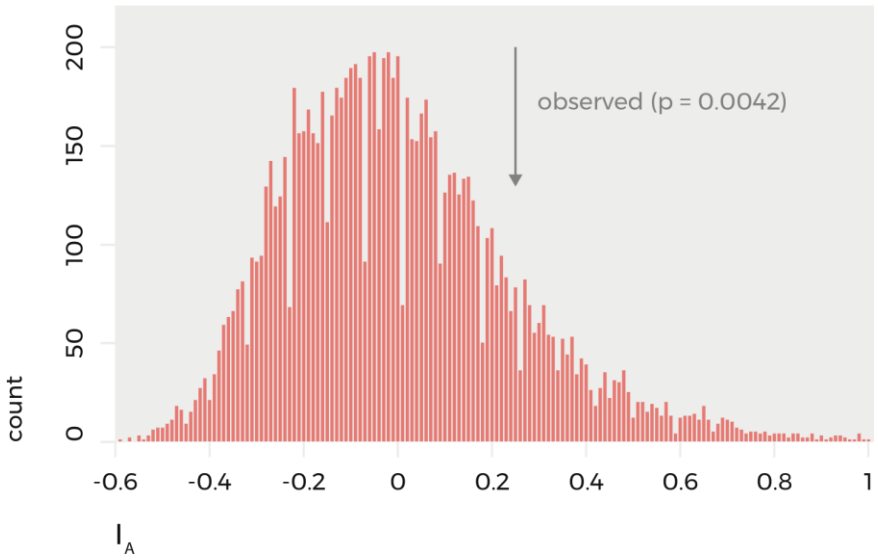


Fig. 9 Histogram of the simulated index of association (I_A) from 10 000 permutations of randomization tests under a null model of allelic recombination; the observed value of I_A is indicated with an arrow

The strongly fixed set of alleles in *T. benhamiae* var. *luteum* indicates low or no gene flow between this cluster and the remaining clusters. Relatively low DW index value (DW = 0.06; Table S5) indicate recent origin of *T. benhamiae* var. *luteum*. On the other hand, high DW values in other taxa indicate long-term isolation due to accumulation of unique alleles (Table S5).

Phenotypic studies

Initially, the phenotype of all isolates was recorded on malt extract agar (MEA). It was observed that the morphotypes within the *T. benhamiae* clade generally corresponded to the clusters delimited by microsatellite analysis. Notable exceptions were the strains showing signs of degeneration (poorly sporulating, white, cottony colonies usually producing no pigments). Such a phenotype is commonly described in

dermatophytes and indicates degeneration, usually caused by long-term strain passaging and preservation (de Hoog et al. 2017). These strains were excluded from further phenotype analyses. At least five strains (if available) from each group were selected, and their phenotypes were analysed on three cultivation media (Fig. 10). Growth rates were recorded at three temperatures (Fig. 11), and micromorphology was measured on MEA (Fig. 12). Cultivation on MEA and potato dextrose agar (PDA) promoted sporulation and pigment production most effectively.

Among the taxa from the *T. benhamiae* clade, the strains of *T. concentricum* and *T. benhamiae* var. *luteum* were characterized by the slowest growth on all media and at all tested temperatures (Fig. 11). No sporulation was observed in the *T. concentricum* strains examined in this study. Overall, poor sporulation, the production of intense yellow pigmentation as the colony reverse colour and the absence of macroconidia and spiral hyphae were characteristic of *T. benhamiae* var. *luteum* (yellow phenotype strains of *T. benhamiae*). All three remaining species from the *T. benhamiae* clade produced both micro- and macroconidia and whitish colonies, usually with a brownish, red-brown or red colony reverse colour

(white phenotype strains of *T. benhamiae*). *Trichophyton benhamiae* var. *benhamiae* grew more rapidly at 25 °C than the other species from this clade (Fig. 11) and exhibited larger microconidia on average (Fig. 12). The obverse colony colour was whitish or showed a brownish tint, and red-brown pigmentation on the reverse side was commonly arranged into sectors (Fig. 10). The growth parameters and micromorphology of *T. japonicum* and *T. europaeum* were very similar (Fig. 11–12), and all strains extensively sporulated.

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

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

MALDI-TOF mass spectrometry

Representative isolates of each species from the *T. benhamiae* clade were analysed using MALDI-TOF mass spectrometry; *T. africanum* isolates were also included for comparison (Fig. 14). All samples could be measured very well and deliv- similar both between and within all groups, and differentiation ered high quality (peak rich) MALDI spectra. In the mass of the groups was not possible within this range. In contrast range between approximately 5900–6200 m/z (as a repre- to this high similarity, several specific peaks could be found sentative example), the MALDI-TOF mass spectra were very for all analysed taxa in the entire mass range of approximately 4000 to 12,000 m/z.The most variable mass range of approximately 4000 to 8000 m/z is shown on Fig. 14. *Trichophyton africanum* significantly differed from all of the samples in many peaks in its spectrum (Fig. 14a).

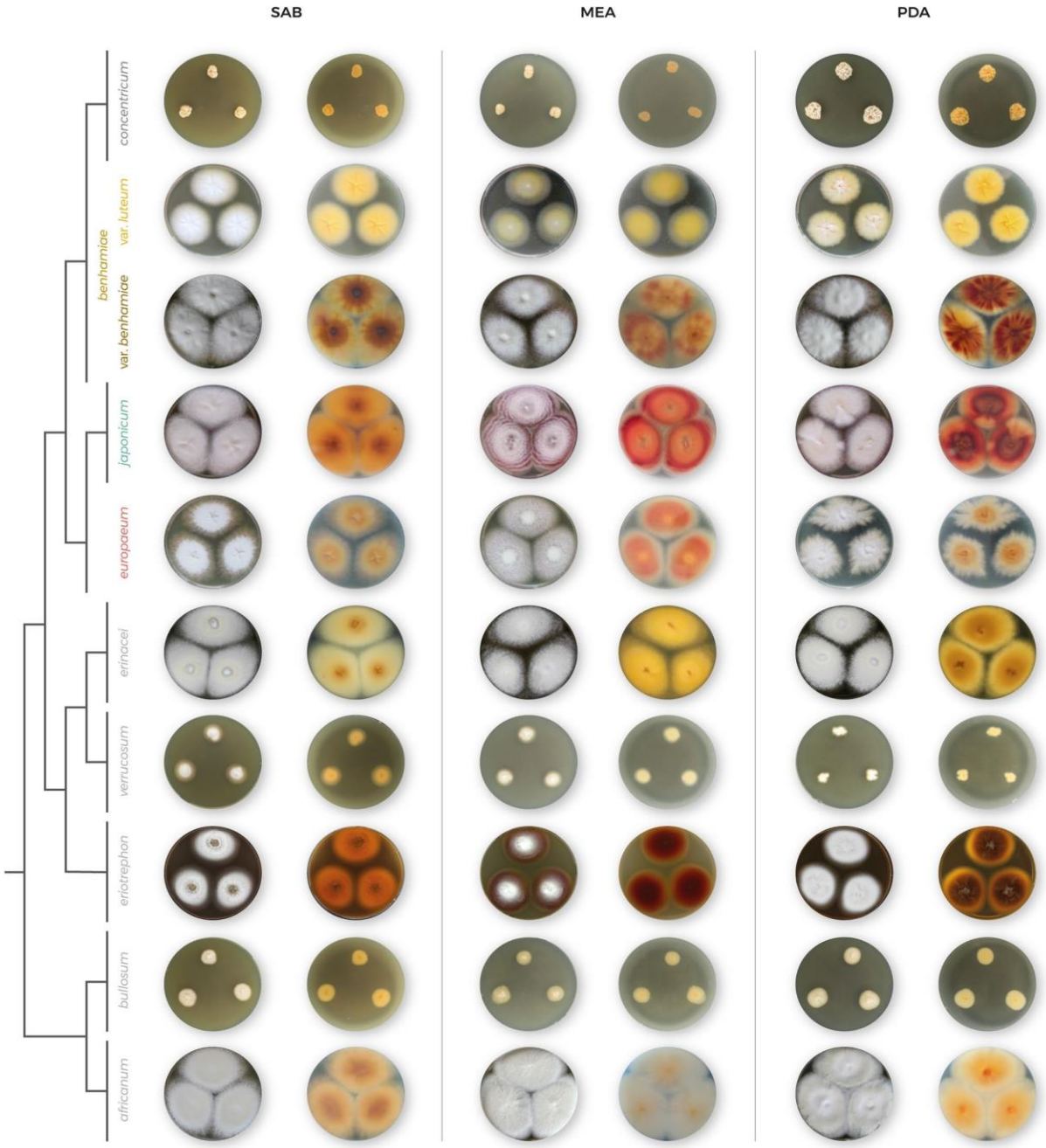


Fig. 10 Overview of the macromorphology of the *Trichophyton benhamiae* complex taxa on three media (SAB, MEA and PDA) cultivated for 14 days at 25 °C

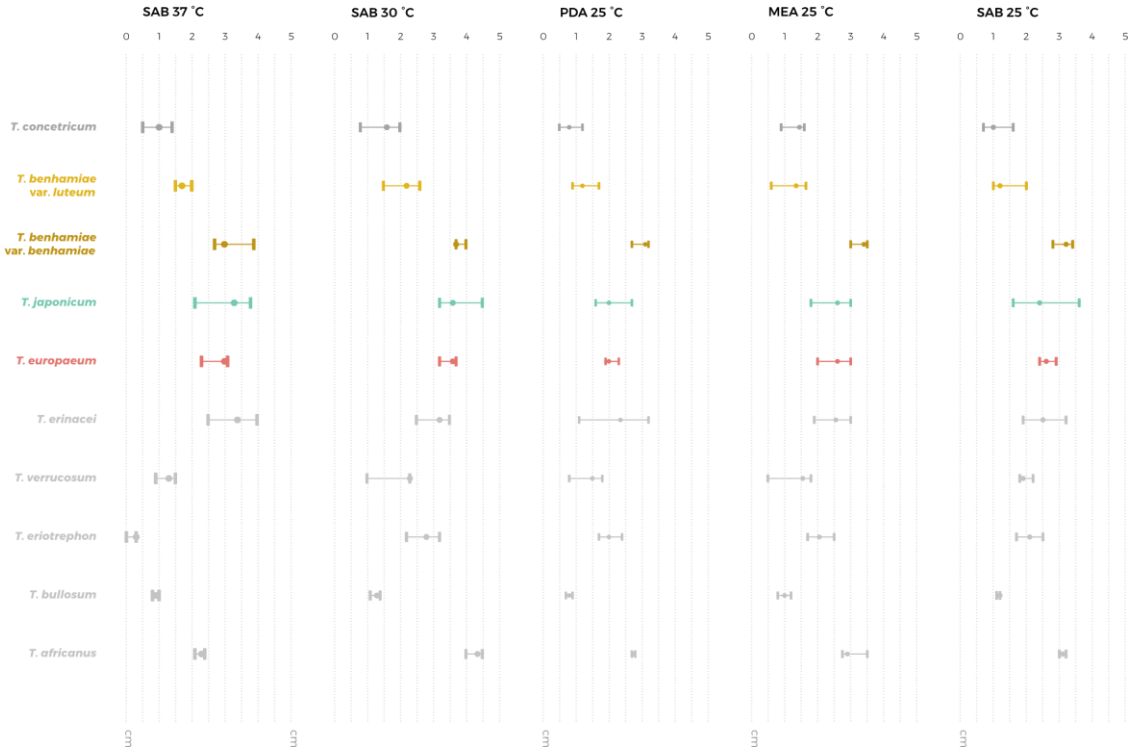


Fig. 11 Growth rates of *Trichophyton benhamiae* complex members on three media (SAB, MEA and PDA) and at three different temperatures (25, 30 and 37 °C, on SAB only) after 7 days of cultivation; circles represent median values and the whiskers span the minimum and maximum values

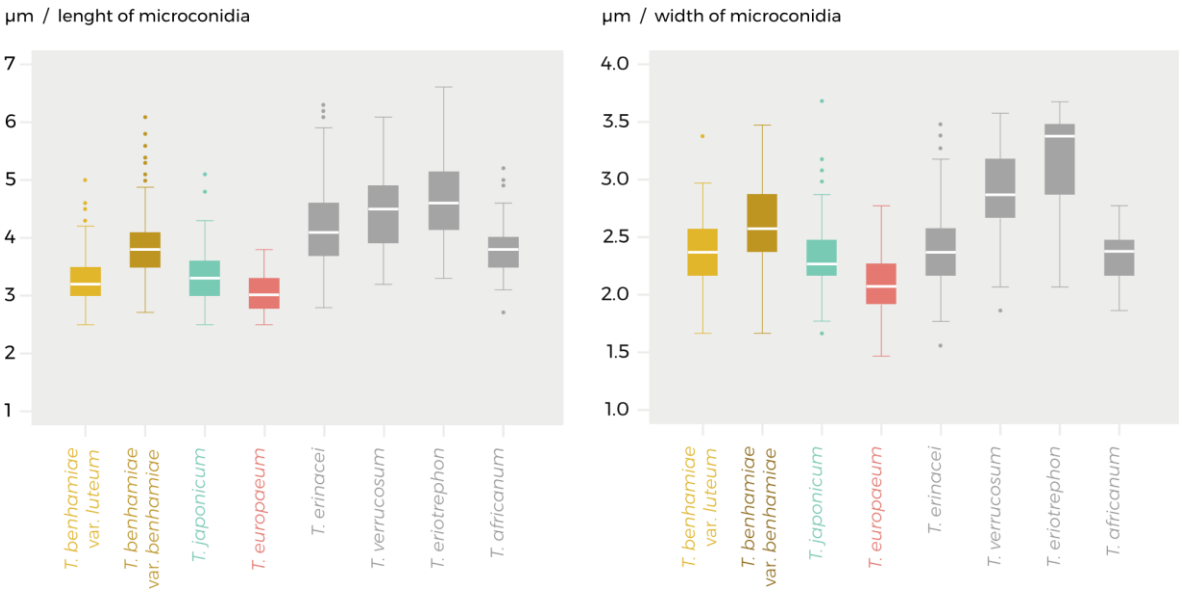


Fig. 12 Length and width of microconidia in taxa belonging to the *Trichophyton benhamiae* complex. The horizontal lines indicate mean value and interquartile range, whiskers span the 5% and 95% percentiles and circles extreme outliers

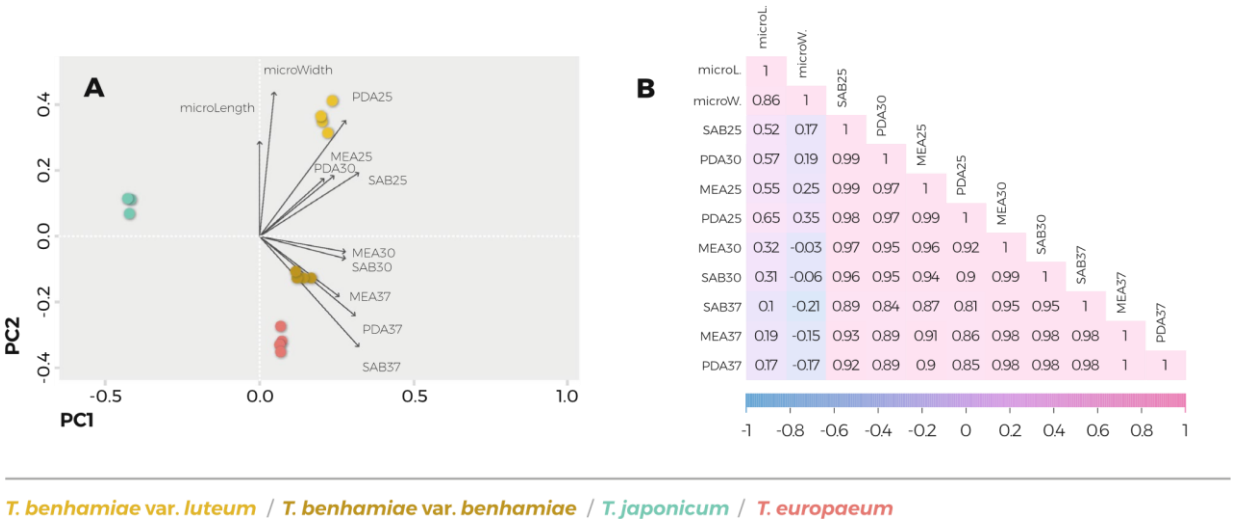


Fig. 13 Principal component analysis (PCA) of morphological characteristics. The two major axes of the plot show all variables, including the growth rates (cultivation on MEA, SAB, and PDA at 25, 30 and 37 °C) and microconidium sizes (mean values of length and width) (**a**). The correlation matrix shows the Pearson correlation coefficients between variables such as growth rates (three different media and temperatures) and microconidia sizes (length and width). A darker colour indicates stronger correlations, which means that all variables within a growth rate or microconidium size group were strongly correlated (**b**), indicating the possibility of reducing the number of variables

Trichophyton benhamiae var. *luteum* and *T. benhamiae* var. *benhamiae* shared peaks at 7150 and 7745 m/z in their mass spectra but different peaks at 4112 and 4680 m/z, which are typical of var. *luteum*, and 6515 and 6530 m/z, which are typical of var. *benhamiae* (Fig. 14c, d). Both mentioned species differ from *T. europaeum* and *T. japonicum* in the absence of a peak at 7150 m/z (data not shown). *Trichophyton europaeum* differed from *T. japonicum* in the presence of a peak at 7745 m/z and the absence of a peak at 7715 m/z (Fig. 14b). *Trichophyton concentricum* differed from both *T. benhamiae* varieties in its peaks at 4770, 6435 and 7145 m/z (Fig. 14d) and also differed from the rest of the samples in several peaks. To prove the general applicability of the here presented MALDI peaks more strains of the mentioned species / varieties should be analyzed in the future and incorporated into the presented MALDI-based differentiation model.

TAXONOMY

Trichophyton benhamiae clade

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

Typus: USA, Missouri, human, L. Ajello, NCDC B765d (*holotype*), PRM 944659 (*epitype*, designated here, MBT 394322) a dried culture derived from strain IHEM 4710; ex-epitype culture IHEM 4710 (= CBS 623.66 = ATCC 16781 = CABIM 124768 = CCF 6484 = CDC X-797 = CECT 2892 = IMI 124768 = IP 1064.74 = NCPF 0410 = RV 23303 = UAMH 2822).

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

Culture characteristics (7 days at 25 °C): Colonies on SAB 28–34 mm diam (⌀ = 32 mm), white (#F2F3F4), velvety to powdery, centrally raised, radially furrowed in some strains, edge diffuse, reverse pale orange yellow (#FAD6A5) to light orange yellow (#FBC97F) in the marginal part, vivid orange (#F38400) to deep brown (#593319) in the center. Colonies on MEA 30–35 mm diam (⌀ = 34 mm), velvety to granular, pale yellow-gray (#C7ADA3) to light yellow (#FAD6A5), umbonate, edge diffuse, reverse pale orange yellow (#FAD6A5) to brilliant orange yellow (#FFC14F), red pigment produced in sectors by some strains—deep reddish orange (#AA381E). Colonies on PDA 27–32 mm diam (⌀ = 30 mm), white (#F2F3F4) to light yellow (#FAD6A5), velvety to granular, centrally raised, occasionally with filamentous sectors, reverse pale orange yellow (#FAD6A5) to brilliant orange

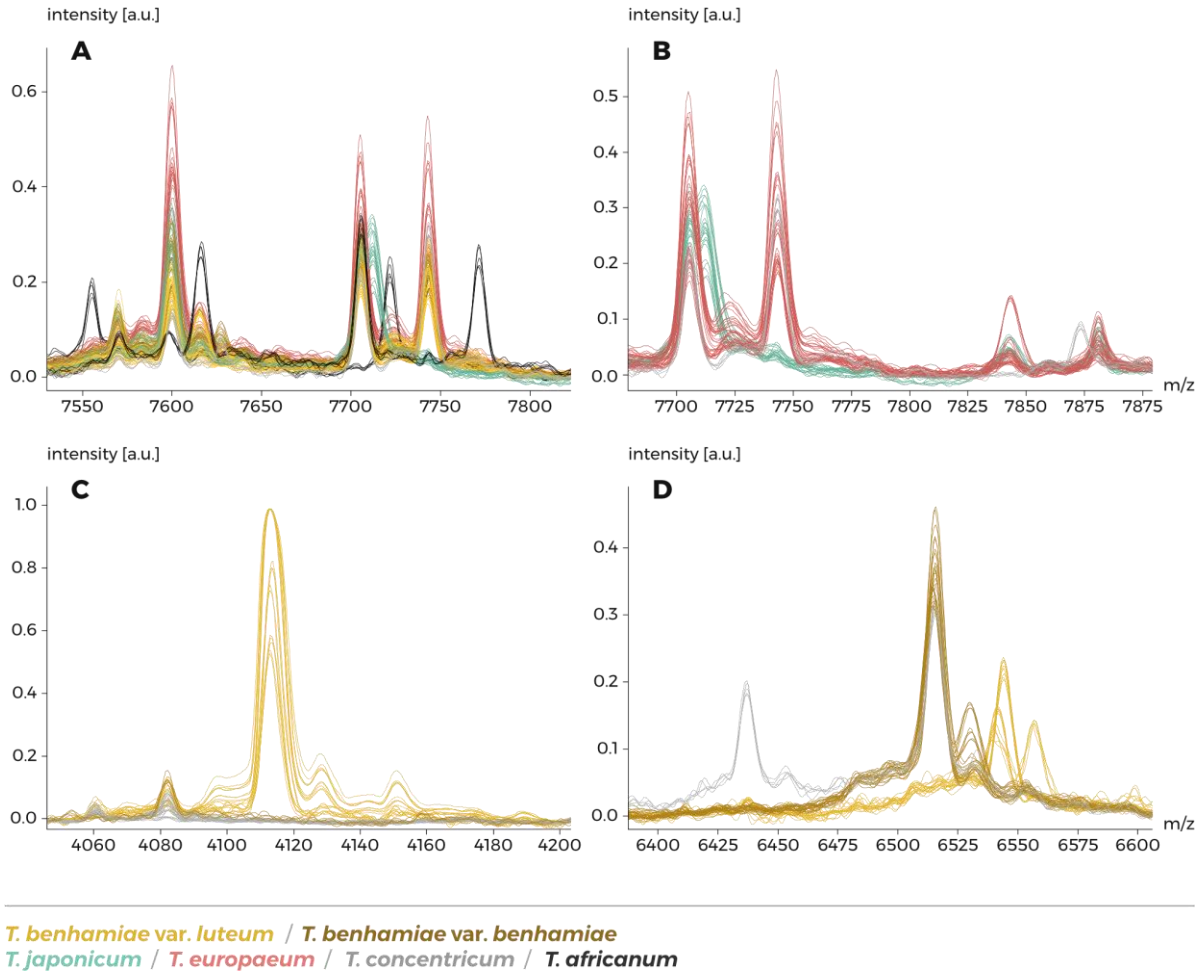


Fig. 14 MALDI-TOF mass spectra in the *Trichophyton benhamiae* clade members; only variable regions are shown. Comparison of spectra in the species of the former Americano-European race (*T. benhamiae* var. *benhamiae* and *T. benhamiae* var. *luteum*, *T. europaeum* and *T. japonicum*), the African race (*T. africanum*) and *T. concentricum* (**a**). Comparison of *T. europaeum*, *T. japonicum* and *T. concentricum* (**b**). Comparison of spectra of *T. concentricum* and two varieties of *T. benhamiae* (**c**, **d**)

yellow (#FFC14F), red pigment produced in sectors by some strains—deep reddish orange pigment (#AA381E). Colonies in 7 days at 30 °C grow faster than at 25 °C: SAB 37–45 mm diam (⌀ = 39 mm); PDA 35–43 mm diam (⌀ = 37 mm); MEA 8–43 mm diam (⌀ = 40 mm). Colonies at 37 °C in 7 d: SAB 27–39 mm diam (⌀ = 30 mm); PDA 30–35 mm diam (⌀ = 34 mm); MEA 30–35 mm diam (⌀ = 33 mm).

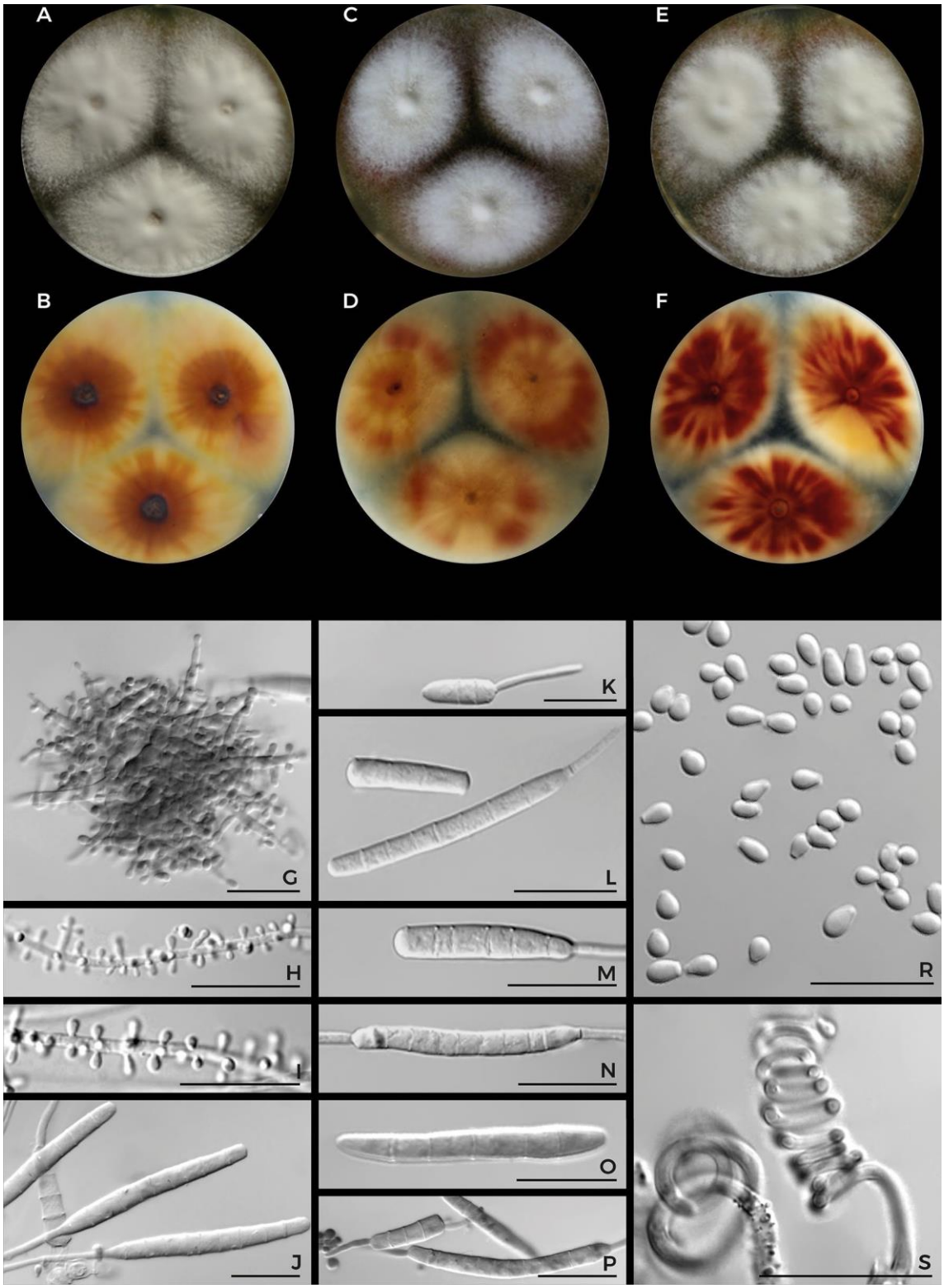


Fig. 15 Macromorphology and micromorphology of *Trichophyton benhamiae* var. *benhamiae*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**a**, **b**), malt extract agar (**c**, **d**) and potato dextrose agar (**e**, **f**). Conidiophores bearing microconidia (**g**–**i**) and macroconidia (**j**); macroconidia (**k**–**p**), frequently with mycelial fragments at one or both ends (**k**–**n**, **p**); microconidia (**r**); spiral hyphae (**s**). Scale bars = 20 μm

Material examined: USA, Missouri, human, L. Ajello (PRM 944659, epitype); ex-epitype culture IHEM 4710 (= CBS 623.66 = ATCC 16781 = CABIM 124768 = CCF 6484 = CDC X-797 = CECT 2892 = IMI 124768 = IP 1064.74 = NCPF 0410 = RV 23303 = UAMH 2822). USA, Urbana, dog, 2009 (USA 3208). USA, Urbana, dog, 2006 (USA 3209); ibid., USA 3216. USA, Urbana, cat, 2006 (USA 3220). USA, Urbana, dog, 2007 (USA 3329). USA, Urbana, dog, 2010 (USA 3350 = CCF 6485); ibid., USA 3355; ibid., USA 3356. USA, Urbana, chinchilla, 2011 (USA 3360 = CCF 6486). USA, Urbana, dog, 2011 (USA 3361). USA, Urbana, unknown source, 1991 (USA 3368). USA, Urbana, unknown source, 1989 (USA 3369). USA, Urbana, unknown source, 1997 (USA 3370). USA, Urbana, unknown source, 2001 (USA 3371). USA, Urbana, unknown source, 1996 (USA 3376). USA, Urbana, unknown source, 1995 (USA 3378). In-vitro, monoascospore isolate, 1970, M. Takashio [IHEM 3287 = RV 26678 = CCF 6483; isolate from cross between IHEM 24908 (ex dog, USA) × IHEM 4710 (ex human, USA)]. In-vitro, monoascospore isolate, 1970, M. Takashio [IHEM 3288 = BER 1464 = DSM 6916 = JS 83-006 = RV 26680 = SM 0104 = VUT 77012 = CCRC 31780 = IAM 12705 = JCM 1886; isolate from cross between IHEM 24908 (ex dog, USA) × IHEM 4710 (ex human, USA)].

Typification Ajello & Cheng (1967) designated the specimen NCDC B765d as a holotype of *Arthroderma benhamiae*, and a dried culture with ascomata was generated by crossing the isolates TM-20 (= ATCC 16781 = IHEM 4710 = CBS 623.66 = CABIM 124768 = CDC X-797 = CECT 2892 = IMI 124768 = IP 1064.74 = NCPF 0410 = RV 23303 = UAMH 2822 = CCF 6484; ex human; MAT1-2-1) × TM-17 (= ATCC 16782 = CBS 624.66 = IHEM 24908 = RV 23302 = CDC X-798 = CECT 2893 = IMI 124769 = NCPF 411 = UAMH 2823; ex dog; MAT1-1-1). Although this specimen exhibits both sexual and asexual morphs in its life cycle, it is not suitable for the purposes of the recent taxonomy for several reasons. First, it is not clear which of the two cultures contained within the type should be considered the ex-holotype culture. Additionally, interspecific hybrids can be induced by crossing opposite mating type strains of unrelated species in vitro as shown in previous studies on dermatophytes (Anzawa et al. 2010; Kawasaki et al. 2009, 2011, 2010), and the deposition of a resultant 'hybrid' type could lead to ambiguities. Because it is not possible to recognize which portion of the holotype belongs to a particular isolate, we designated an epitype PRM 944659 (dried culture) derived from the strain IHEM 4710.

Distribution and ecology: Trichophyton benhamiae var. *benhamiae* is a zoophilic dermatophyte, and isolates examined in this study originated from dogs (n = 8), cats (isolate USA 3220), chinchillas (isolate USA 3360) and unknown hosts (n = 6). Previously reported cases of human infections were probably transmitted from animals (Ajello and Cheng 1967). Another important host of this pathogen is probably the North American porcupine (*Erethizon dorsatum*) (Needle et al. 2019; Takahashi et al. 2008), a close relative of the guinea pig (*Cavia porcellus*). Isolates from the North American porcupine exhibited ITS rDNA identical to that of *T. benhamiae,* and their morphology showed characteristics

typical of *T. benhamiae* var. *benhamiae* (Needle et al. 2019; Takahashi et al. 2008). All strains examined here were collected in North America (the in vitro-derived isolates IHEM 3287, IHEM 3288, IHEM 4710 were also based on strains of American origin). A recently reported Chinese case of tinea faciei, likely contracted from fox, was probably also caused by *T. benhamiae* var. *benhamiae* based on the ITS sequence and morphology of the isolate (Tan et al. 2020).

Notes: The macromorphology of *T. benhamiae* var. *benhamiae* most closely resembles those of *T. europaeum, T. japonicum* and *T. mentagrophytes* in the production of a redbrown pigment on the reverse side of colonies and abundant microconidia. It differs from *T. europaeum* and *T. japonicum* in its host spectrum and higher growth rates, especially on MEA and PDA at 25 °C (Fig. 11). Macroconidia of *T. benhamiae* var. *benhamiae* are usually more abundantly produced compared to *T. europaeum* and *T. japonicum*, and they are most frequently cylindrical or elongated fusiform with terminal fragments of vegetative hyphae. Closely related *T. concentricum* differs significantly in its ecology. It is an anthropophilic species occurring in tropical regions, grows very slowly, produces cerebriform colonies without red-brown pigment on the colony reverse and usually does not sporulate. *Trichophyton behamiae* var. *luteum* is also strikingly different in its host spectrum (mostly guinea pigs), distribution (mainly Europe) and morphology (slow growth, yellow pigmentation, relatively poor sporulation, absence of macroconidia). *Trichophyton benhamiae* var. *benhamiae* does not produce intense yellow pigment on SAB supplemented with chloramphenicol and cycloheximide and MEA, in contrast to *T. benhamiae* var. *luteum*. The ratio of MAT11-1 and MAT1-2-1 strains was 14:5.

Trichophyton benhamiae (Ajello & S.L. Cheng) Y. Gräser & de Hoog [Index Fungorum 356: 2. 2018] **var. *luteum*** Cmokova & Hubka, **var. nov.—**MycoBank MB835887;
Fig. 16

Etymology: Refers to the bright yellow colony reverse produced on all examined media.

Typus: SWITZERLAND, Lausanne, University Hospital Vaudois, dermatophytosis in human, arm skin (tinea corporis), 2009, M. Monod, PRM 944414 (holotype); exholotype culture IHEM 25068 (= CCF 6500).

Vegetative hyphae smooth, septate, hyaline, 1–3.5 µm diam (mean ± sd: 1.9 ± 0.5). *Conidiophores* branched in a pyramidal (grape-like) pattern, sometimes poorly differentiated from vegetative hyphae, unbranched or poorly branched, conidia sessile or born on short lateral branches.

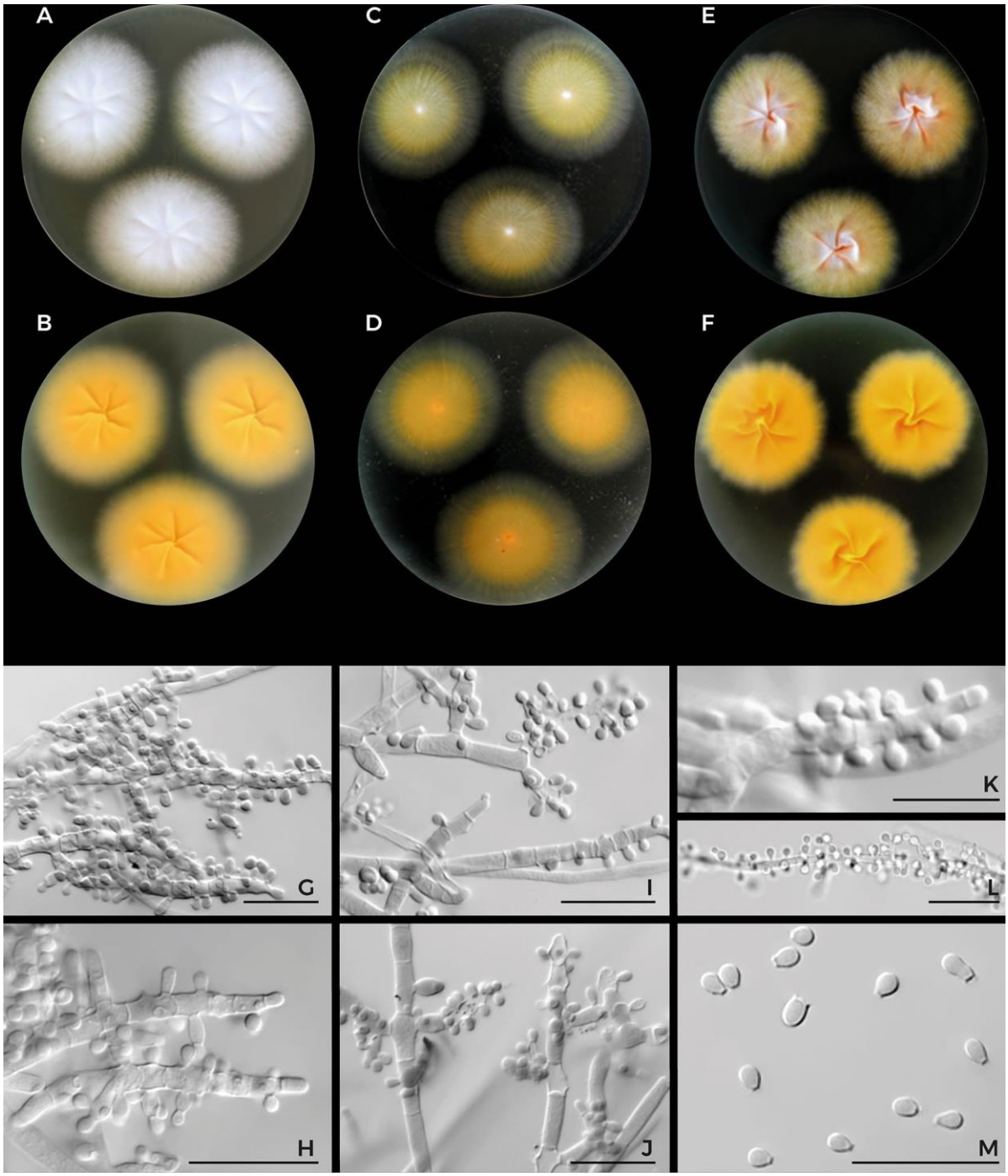


Fig. 16 Macromorphology and micromorphology of *Trichophyton benhamiae* var. *luteum*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**a**, **b**), malt extract agar (**c**, **d**) and potato dextrose agar (**e**, **f**). Conidiophores bearing microconidia (**g**–**l**); microconidia (**m**). Scale bars = 20 μm

Microconidia sparse to abundant, pyriform, less commonly clavate, 2.5–4.9 (3.2 ± 0.4) × 1.5–3.4 (2.1 ± 0.3) µm. *Macroconidia* not observed in any of the isolates examined. *Chlamydospores* were not observed. *Spiral hyphae* not observed. *Sexual morph* unknown.

Culture characteristics (7 days at 25 °C): colonies on SAB 10–20 mm diam (⌀ = 13 mm), white (#F2F3F4) to yellowish white (#F0EAD6), velvety, flat with radially furrowed center, edge filliform, reverse vivid yellow (#F3C300). Colonies on MEA 6–17 mm diam (⌀ = 12 mm), pale yellow (#F3E5AB), filamentous, in large extent submerged, flat, edge filliform, reverse light yellow (#F8DE7E) to vivid yellow (#F3C300). Colonies on PDA 9–17 mm diam (⌀ = 13 mm), light yellow (#F8DE7E) to pale yellow (#F3E5AB), velvety, flat, radially furrowed, edge filliform, reverse brilliant orange yellow (#FFC14F) to vivid yellow (#F3C300). Colonies at 30 °C in 7 d: SAB 15–26 mm diam (⌀ = 21 mm); PDA 18–22 mm diam (⌀ = 21 mm); MEA 21–22 mm diam (⌀ = 22 mm). Colonies at 37 °C in 7 d: SAB 15–20 mm diam (⌀ = 18 mm); PDA 10–17 mm diam (⌀ = 12 mm); MEA 11–13 mm diam (⌀ = 11 mm).

Material examined: Switzerland, Lausanne, University Hospital Vaudois, dermatophytosis in human, arm skin (tinea corporis), 2009, M. Monod (PRM 944414, holotype, dried culture; PRM 944415, isotype); ex-holotype culture IHEM 25068 = CCF 6500. Japan, common degu, 2012 (NUBS 13001). Switzerland, Lausanne, University Hospital Vaudois, human skin, 2009, M. Monod (IHEM 25066). Czechia, Prague, guinea pigs (*Cavia porcellus*), 2014, J. Koubová (KOUB 23); ibid., KOUB 51; ibid., KOUB 77. Germany, Berlin, dermatophytosis in human, 2010 (BER 24); ibid., BER 211; ibid., BER 212; ibid., BER 213. Czechia, České Budějovice, dermatophytosis in human, 2012 (D126); ibid., D295; ibid., D375; ibid., D417; ibid., D521. Germany, Mölbis, dermatophytosis in human, 2015 (DE 200156); ibid., DE 200351; ibid., DE 200465. Belgium, Brussels, dermatophytosis in human, 2012 (IHEM 25744 = CCF 6476); ibid., IHEM 25743; ibid., IHEM 25742 = CCF 6474; ibid., IHEM 25466; ibid., IHEM 25745. Czechia, Prague, dermatophytosis in human, 2012 (CCF 4849); ibid., CCF 4850; ibid., CCF 4851; ibid., CCF 4852. All 236 strains examined in this study are listed in Table S1.

Distribution and ecology: Trichophyton benhamiae var. *luteum* is a zoophilic species with the guinea pig as the main host (Hubka et al. 2018d). It is widely distributed in Europe, but it has also been detected in common degu (*Octodon degus*) in Japan (Hiruma et al. 2015) and was recently isolated from human dermatophytosis in Brazil (de Freitas et al. 2019; Grisólia 2019) and Iraq (S. Uhrlaß, unpublished data) (Table S10).

The European strains of *T. benhamiae* var. *luteum* (n = 236) examined here were predominantly obtained from humans (~ 72% from females and ~ 28% from males, median age 12 years) who mostly reported contact with guinea pigs; the remaining strains were recovered from animals (guinea pigs and common degu) (Table S1). The human infections mostly manifested as highly inflammatory tinea corporis, tinea faciei and tinea capitis (Fig. 17). By contrast, infected animals were mostly

symptomless. Symptomatic guinea pigs usually showed localized lesions with scaling and crusting or alopecia located predominantly on the head, less frequently on the other body parts (Fig. 17). Green fluorescence of infected tissues may be observed under Wood's light in some strains, similar to *M. canis* (Skořepová et al. 2014). Only the MAT1-1-1 idiomorph was detected in the *T. benhamiae* var. *luteum* isolates examined here.

Notes: The macromorphology of *T. benhamiae* var. *luteum* resembles that of *Microsporum canis* in the production of intense yellow pigments. However, *M. canis* usually produces abundant spindle-shaped macroconidia, which are absent in *T. benhamiae* var. *luteum*. The differentiation of sterile *M. canis* isolates may be more difficult but is possible according to its higher growth parameter values. In addition, these species differ in their main hosts, which are cats and dogs in *M. canis* and guinea pigs in *T. benhamiae* var. *luteum*. The closely related anthropophilic species *T. concentricum* differs in its ecology, colony characteristics (no yellow pigment produced) and microscopic characteristics (usually no sporulation). Other taxa from the *T. benhamiae* clade differ in showing higher growth rates (Fig. 11), the production of red/brown pigments and the production of macroconidia, which are absent in *T. benhamiae* var. *luteum*. In addition to these differences, *T. benhamiae* var. *luteum* can be clearly distinguished from *T. benhamiae* var. *benhamiae* and other species in the *T. benhamiae* clade by microsatellite data (Figs. 5–6) and MALDI-TOF MS spectra (Fig. 14).

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

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

Culture characteristics (7 days at 25 °C): Colonies on SAB 6–16 mm diam (⌀ = 11 mm), pale orange yellow (#FAD6A5) to pale yellowish pink (#ECD5C5), membranous to slightly velvety, raised, umbonate or cerebriform, deeply furrowed, edge filiform or lobate, reverse light orange yellow (#FBC97F). Colonies on MEA 9–16 mm diam (⌀ = 15 mm), pale orange yellow (#FAD6A5) to pale yellowish pink (#ECD5C5), membranous to slightly velvety, umbonate, edge filiform, reverse light orange yellow (#FBC97F) to brilliant orange yellow (#FFC14F), vivid yellow (#F3C300) in narrow centre. Colonies on PDA 5–12 mm diam (⌀ = 11 mm), pale orange yellow (#FAD6A5) to pale yellowish pink (#ECD5C5), membranous, raised, deeply furrowed to cerebriform, edge irregular

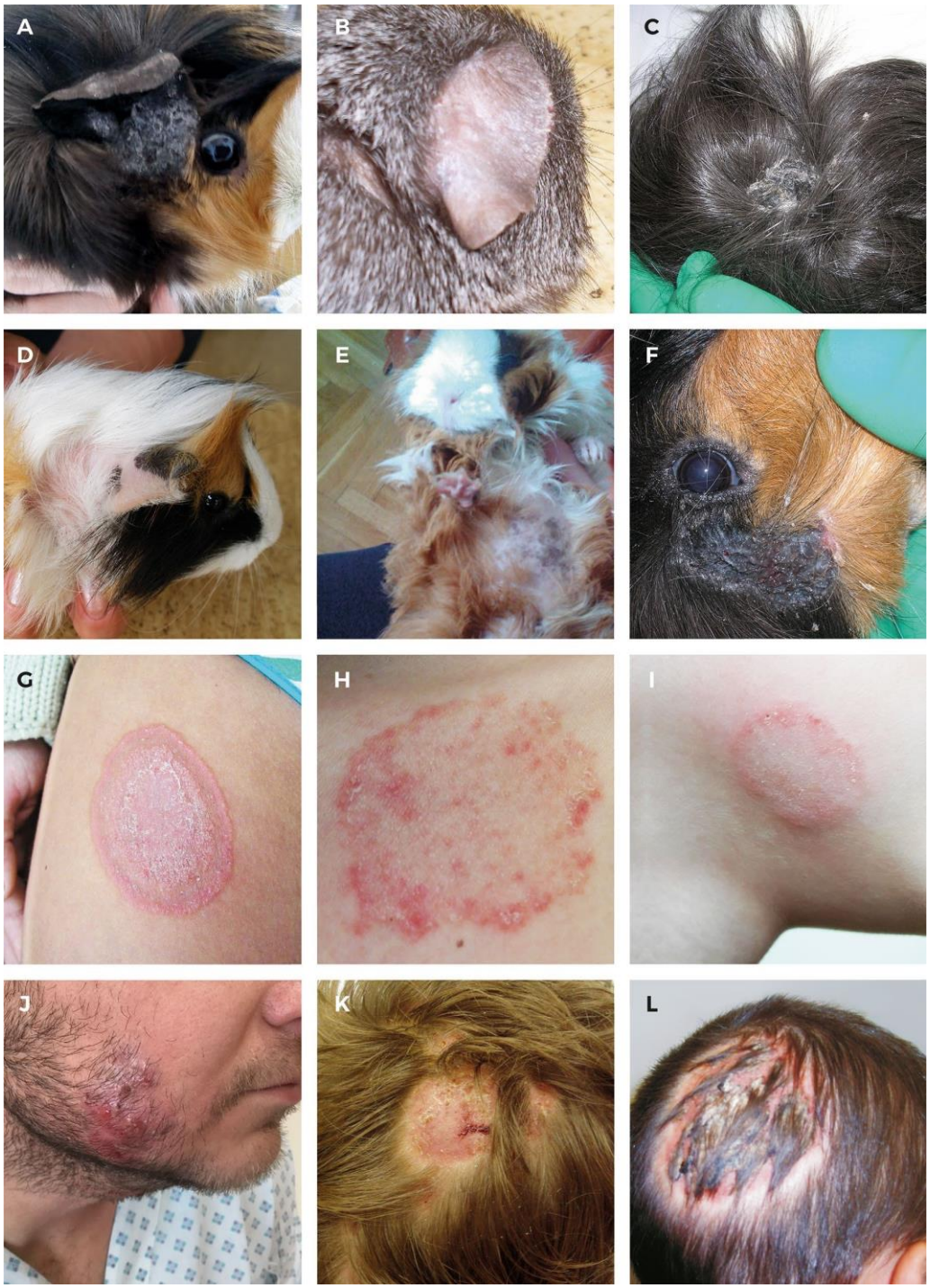


Fig. 17 Clinical presentation of infections caused by *Trichophyton benhamiae* clade species in guinea pigs and humans. Guinea pigs: area of alopecia with scaling located in the temporal area (**a**); areas with scaling on the ear (**b**); area of alopecia with scaling located on the back (**c**); itchy area of alopecia behind the ear (**d**) and on the guinea pig's abdomen (**e**); weeping lesion under the eye (**f**). Zoonotic infections in humans: tinea corporis located on the thigh (**g**) and chest (**h**), tinea faciei (**i**), tinea barbae (**j**), tinea capitis profunda (**k, l**)

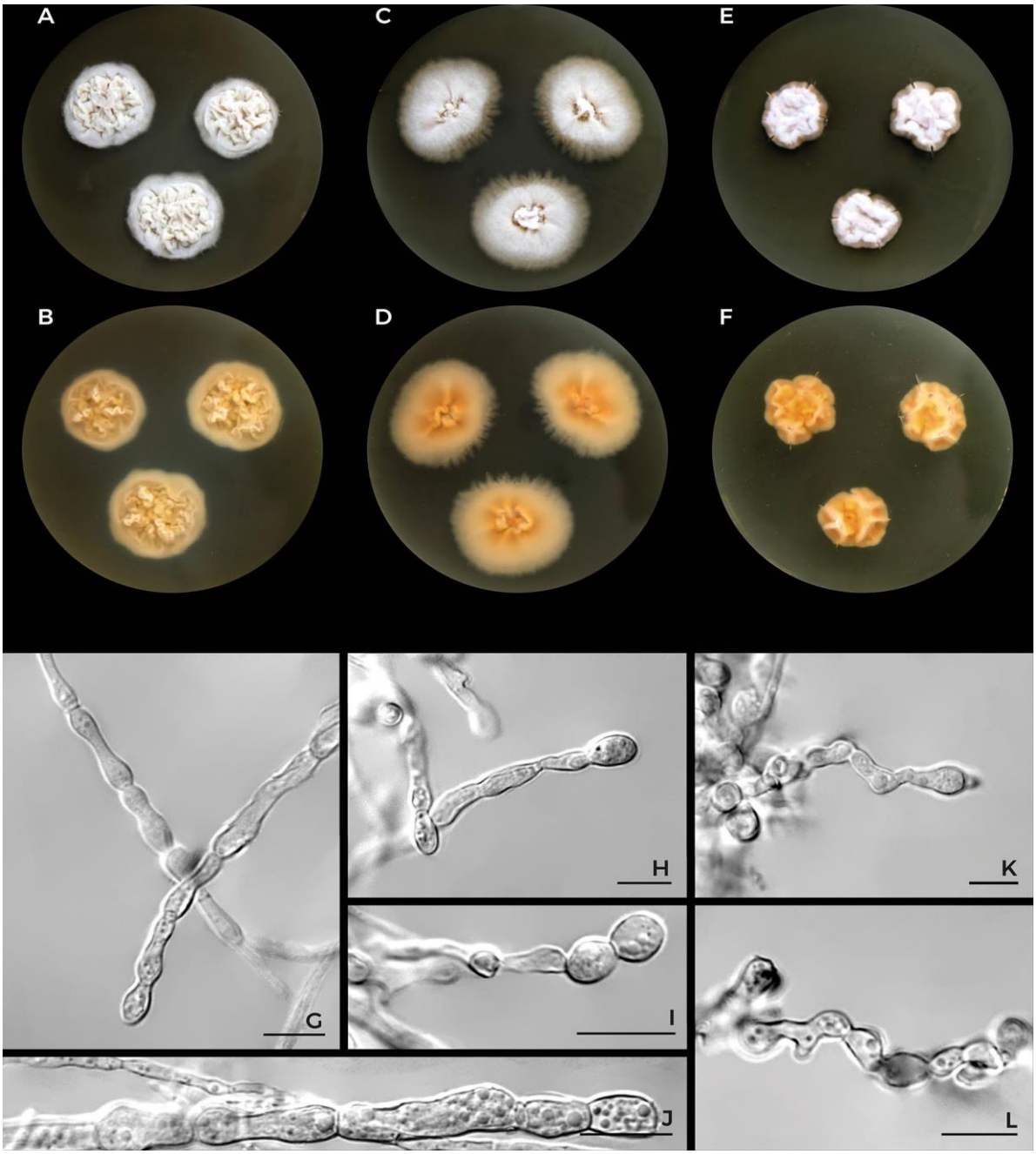


Fig. 18 Macromorphology and micromorphology of *Trichophyton concentricum*. Colonies after three weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**a**, **b**), malt extract agar (**c**, **d**) and potato dextrose agar (**e**, **f**). Vegetative hyphae (**g**–**l**), frequently consisting of inflated cells and containing intercalary or terminal chlamydospores (**h**, **i**), occasionally proliferating in a zigzag pattern (**k**, **l**). Scale bars = 20 µm

to lobate, reverse light orange yellow (#FBC97F) to brilliant orange yellow (#FFC14F), vivid yellow (#F3C300) in narrow centre. Colonies at 30 °C in 7 d: SAB 8–20 mm diam (⌀ = 16 mm); MEA 8–15

mm diam (⌀ = 13 mm); PDA 10–14 mm diam (⌀ = 11 mm). Colonies at 37 °C in 7 d: SAB 5–14 mm diam (⌀ = 10 mm); MEA 5–13 mm diam (⌀ = 10 mm); PDA 5–13 mm diam (⌀ = 9 mm).

Material 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; Bonifaz and Vazquez-Gonzalez 2011; Pihet et al. 2008).

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 (Fig. 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 macoconidia has been observed by some authors (Pihet et al. 2008; Rippon 1988), while favic chandeliers and pectinate hyphae (“antler” tips) are more frequently reported (Bonifaz et al. 2004; Dvořák and Otčenášek 1969). We did not observe these structures in any of the isolates examined.

Trichophyton europaeum Cmokova & Hubka, ***sp. nov.***—
MycoBank MB835888; Fig. 19

Etymolog: Refers to the origin of the examined strains.

Typus: SWITZERLAND, Lausanne, guinea pig (*Cavia porcellus*), 2008, M. Monod, PRM 944419 (holotype); exholotype culture IHEM 22725 (= CCF 6499).

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

Culture characteristics (7 days at 25 °C): Colonies on SAB 24–29 mm diam (⌀ = 25 mm), White (#F2F3F4), velvety to floccose, flat, in some strains with radially wrinkled or elevated center, edge filiform, diffuse or entire, reverse brilliant yellow (#FADA5E), to deep orange yellow (#C98500). Colonies on MEA 20–30 mm diam (⌀ = 26 mm), white (#F2F3F4) to light yellow (#F8DE7E), velvety, floccose to coarsely granular, flat with an umbonate center, edge entire to diffuse, reverse in shades of brown [strong orange yellow (#EAA221) to deep orange (#BE6516)] or red [vivid reddish orange (#E25822) to vivid red (#BE0032)]. Colonies on PDA 19–23 mm diam (⌀ = 21 mm), white (#F2F3F4) to light yellow (#F8DE7E), velvety, floccose to coarsely granular, flat with an umbonate center, edge irregular, lobate dendritic, reverse yellow (#F3C300) in the marginal part, strong orange yellow (#EAA221) to deep orange (#F38400) in the center. Colonies at 30 °C in 7 d: SAB 32–37 mm diam (⌀ = 35 mm); PDA 29–31 mm diam (⌀ = 30 mm); MEA 32–39 mm diam (⌀ = 36 mm). Colonies at 37 °C in 7 d: SAB 23–31 mm diam (⌀ = 28 mm); PDA 24–31 mm diam (⌀ = 29 mm); MEA 20–30 mm diam (⌀ = 27 mm).

Material examined: Switzerland, Lausanne, guinea pig (*Cavia porcellus*), 2008, M. Monod (PRM 944419, holotype, dried culture); ex-holotype culture IHEM 22725 (= CCF 6499). France, Lyon, guinea pig, 1963 (IHEM 25139 = CBS 806.72 = RV 14387 = ATCC 28061 = CCF 6480). Switzerland, Lausanne, human dermatophytosis (contact with guinea pig), 2002, M. Monod (IHEM 20159 = CBS 112370); ibid., IHEM 25062 = CCF 6477. Switzerland, Lausanne, human dermatophytosis (contact with guinea pig), 2007, M. Monod (IHEM 25064 = CCF 6478). Switzerland, Lausanne, tinea corporis (contact with guinea pig), 2010, M. Monod (IHEM 25075). Switzerland, Lausanne, tinea faciei, 2011, Monod (HEM 25076). Switzerland, Lausanne, guinea pig, 2002, M. Monod (IHEM 22723). Czechia, Malhotice, toenail (onychomycosis), 2012, S. Dobiášová (CCF 4917). Czechia, Prague, human dermatophytosis (tinea faciei), 2012, M. Skořepová (CCF 4848). Czechia, Bylany, dermatophytosis in human (tinea corporis), M. Skořepová (CCF 4853). All 40 strains of *T. europaeum* examined in this study are listed in Table S1.

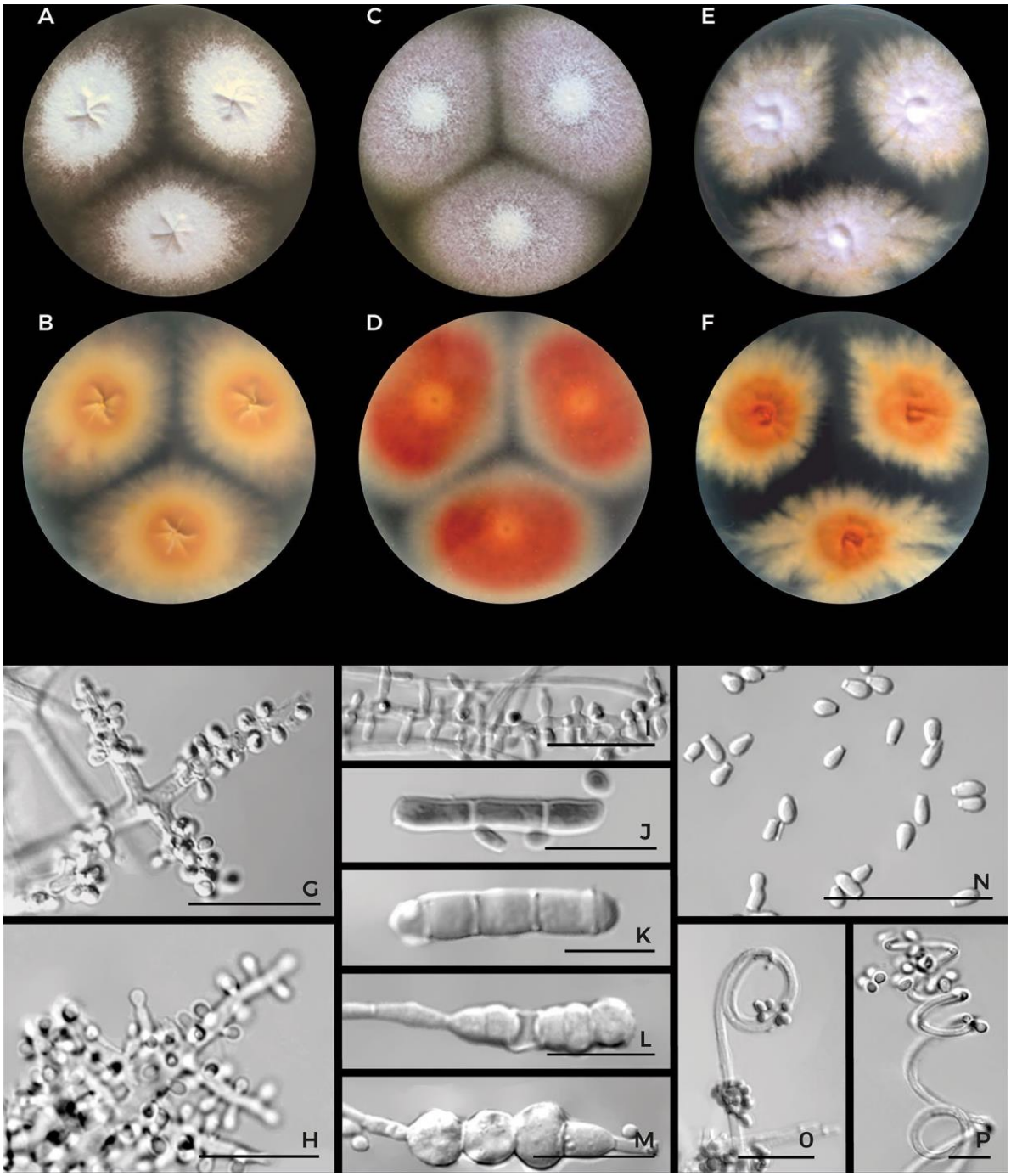


Fig. 19 Macromorphology and micromorphology of *Trichophyton europaeum*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**a**, **b**), malt extract agar (**c**, **d**) and potato dextrose agar (**e**, **f**). Conidiophores bearing microconidia (**g**–**i**); macroconidia (**j**–**m**); microconidia (**n**); spiral hyphae (**o**, **p**). Scale bars = 20 μm

Distribution and ecology: Trichophyton europaeum is a zoophilic species that is widely distributed in guinea pigs in Europe (Fréalle et al. 2007; Fumeaux et al. 2004; Sabou et al. 2018; Symoens et al. 2013) but is less prevalent than *T. benhamiae* var. *luteum*. The species has also been reported from fox in Poland (Ziółkowska et al. 2015), guinea pigs in Japan (Takeda et al. 2012) and human dermatophytosis in Iran (Rezaei-Matehkolaei et al. 2016). Dermatophytosis in horses reported in Egypt is an unusual finding (Tartor et al. 2016).
The European strains of *T. europaeum* (n = 41) examined here were predominantly obtained from humans (~ 80% from females and ~ 20% from males, median age 12 years) who mostly reported contact with guinea pigs (66%), and the remaining strains were recovered from guinea pigs or dogs (Table S1). The infections mostly manifested as tinea corporis (79%) and tinea faciei (21%). Only the MAT1-2-1 idiomorph was detected in the *T. europaeum* isolates examined here, with the exception of the IHEM 25139 strain.

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

Trichophyton japonicum Cmokova & Hubka, ***sp. nov.***— MycoBank MB835889; Fig. 20

Etymology: Refers to the origin of the majority of the examined strains.

Typus: SPAIN, human, 1963, P. Miguens, PRM 944416 (holotype); ex-holotype culture IHEM 17701 = ATCC 28063 = CBS 807.72 = CECT 2894 = RV 14988 = CCF 6481.

Vegetative hyphae smooth, septate, hyaline, 1.5–4 µm diam (mean ± sd: 2.5 ± 0.6). *Conidiophores* usually poorly differentiated from hyphae and represented by conidiogenous hyphae with sparse to numerous short lateral branches; conidiophores branched in a pyramidal (grape-like) pattern relatively rare. *Microconidia* abundant, born terminally on hyphae, pyriform to clavate, 2.5–5 (3.2 ± 0.4) × 1.5–3.6 (2.3 ± 0.3) µm. *Macroconidia* rare to abundant, born terminally on hyphae, sparse to abundant depending on the isolate, consisting of 3–8(–12) cells (median = 5), 11–79 (55.2 ± 12.4) × 5–11 (6.8 ± 1.5) µm, elongated, cigar-shaped, clavate, with a tapering rounded apex and truncate end, macroconidia consisting of irregular and bloated cells common, long macroconidia easily disintegrate into cylindrical fragments. *Chlamydospores* present. *Spiral hyphae* absent to sparse in 14-days-old colonies. *Sexual morph* unknown.

Culture characteristics (7 days at 25 °C): Colonies on SAB 16–36 mm diam (⌀ = 23 mm), white (#F2F3F4) to pale yellowish pink (#ECD5C5), velvety to floccose, flat with sligtly elevated and furrowed center, edge entire to diffuse, reverse light orange (#FAB57F) to vivid orange yellow (#F6A600) in the marginal part, in some strains deep orange yellow (#C98500) center. Colonies on MEA 18–30 mm diam (⌀ = 26 mm), white (#F2F3F4), light yellow (#F8DE7E) to pale yellowish pink (#ECD5C5), floccose to granular, flat, sometimes with an umbonate center, frequently with concentric ring pattern, margin entire to diffuse, reverse deep orange (#BE6516), strong reddish brown (#882D17) to vivid red (#BE0032). Colonies on PDA 16–27 mm diam (⌀ = 23 mm), white (#F2F3F4) to pale yellowish pink (#ECD5C5), floccose to granular, occasionally with cottony sectors, flat or umbonate, margin entire, reverse deep orange (#BE6516), strong reddish brown (#882D17) to vivid red (#BE0032). Colonies at 30 °C in 7 d: SAB 32–45 mm diam (⌀ = 38 mm); MEA 28–37 mm diam (⌀ = 33 mm); PDA 26–35 mm diam (⌀ = 30 mm). Colonies at 37 °C in 7 d: SAB 21–38 mm diam (⌀ = 26 mm); MEA 32–37 mm diam (⌀ = 35 mm); PDA 30–35 mm diam (⌀ = 33 mm).

Material examined: Spain, human, 1963, P. Miguens (PRM 944416, holotype, dried culture; PRM 944417, isotype); ex-holotype culture IHEM 17701 = ATCC 28063 = CBS 807.72 = CECT 2894 = RV 14988 = CCF 6481). Belgium, dog, 1971, De Vroey (IHEM 4030 = ATCC 28067 = CBS 809.72 = RV 28105 = CCF 6498). Japan, rabbit, 2009 (NUBS 09011). Japan, Saitama, human, 2000 (VUT 00003–2). Japan, Saitama, rabbit, 1999 (VUT 00002). Japan, Saitama, rabbit, 2000 (VUT 00003). Japan, human, 2013 (NUBS 12001). Japan, Hyogo, rabbit, 1997 (VUT 97010 = CCF 6489). Japan, unknown source (JPN3 = CCF 6487). Japan, unknown source (JPN6). Japan, human, unknown (NUBS 13002 = CCF 6488). Czechia, human, tinea corporis, 2013, N. Mallátová (D 35). Czechia, human, tinea corporis, 2011, S. Dobiášová (DMF 3061). Czechia, human, tinea corporis, 2012, S. Dobiášová (DMF 2446); ibid., DMF 3031. Czechia, human, tinea corporis, 2013, S. Dobiášová (DMF 1658). Czechia, guinea pig (*Cavia porcellus*), 2014, J. Koubková (KOUB 63). Czechia, Pardubice, human, tinea

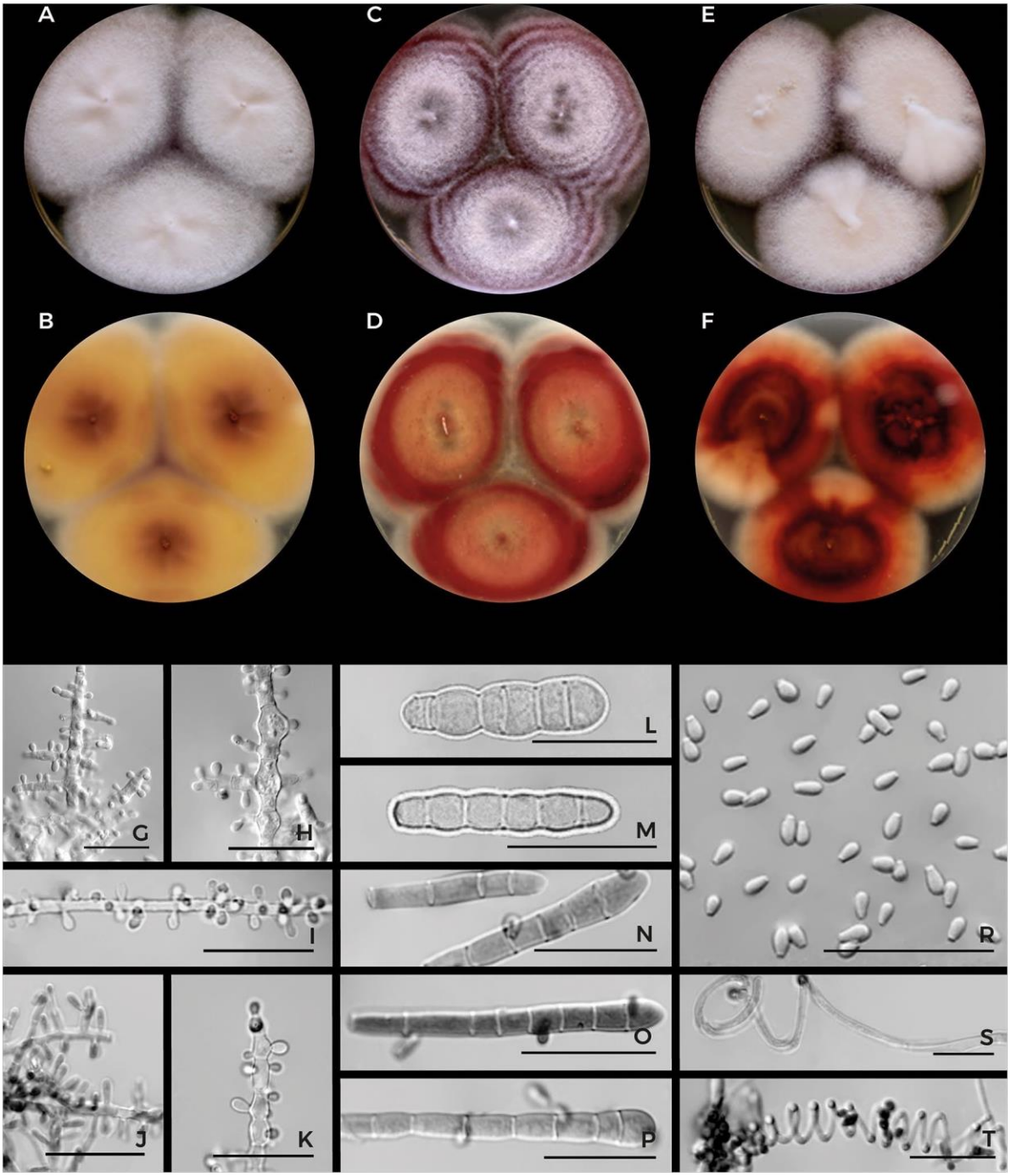


Fig. 20 Macromorphology and micromorphology of *Trichophyton japonicum*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**a**, **b**), malt extract agar (**c**, **d**) and potato dextrose agar (**e**, **f**). Conidiophores bearing microconidia (**g**–**k**); macroconidia (**l**–**p**); microconidia (**r**); spiral hyphae (**s**, **t**). Scale bars = 20 μm

corporis, 2011, K. Mencl (ME 961). Czechia, Prague, human, tinea corporis, 2012, P. Lysková (PL 1773).

Distribution and ecology: Trichophyton japonicum is a zoophilic species occurring mostly in rabbits and guinea pigs. The species is widely distributed in Japan (mostly in rabbits) (Kimura et al. 2015; Takeda et al. 2012). In Europe it occurs mostly in guinea pigs and less frequently in rabbits and other hosts (Table S10). In guinea pigs it is less common than *T. benhamiae* var. *luteum* and *T. europaeum* (see discussion). It has also been detected in Iran (GenBank JX413540), Oman (Al-Hatmi 2010), Taiwan (Wang and Sun 2018), Thailand (Vu et al. 2019) and South Korea (P.-L. Sun, pers. comm.) (Table S10).

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

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

***Trichophyton erinacei* clade**

Trichophyton erinacei (J.M.B. Sm. & Marples) Quaife, J. Clin. Pathol. 19: 178. 1966—Fig. 21

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

Culture characteristics (7 days at 25 °C): Colonies on SAB 19–32 mm diam (⌀ = 29 mm), white (#F2F3F4) to light orange yellow (#F3E5AB) in the centre, flat, finely to coarsely granular, edge diffuse, reverse light orange yellow (#F3E5AB) to vivid yellow (#FADA5E), deep reddish brown (#882D17) in the centre. Colonies on MEA 19–30 mm diam (⌀ = 25 mm), white (#F2F3F4), flat, finely to coarsely granular, edge diffuse, reverse light orange yellow (#F3E5AB) to vivid reddish orange

(#F38400). Colonies on PDA 11–32 mm diam (⌀ = 24 mm) white (#F2F3F4) to pale orange yellow (#F3E5AB), flat to slightly raised in the center, finely to coarsely granular (velvety to cottony in some strains), edge diffuse (irregular or submerged in some strains), light orange yellow (#F3E5AB) to vivid yellow (#F3C300), frequently deep reddish brown (#882D17) in the centre. Colonies at 30 °C in 7 d: SAB 25–35 mm diam (⌀ = 31 mm); MEA 39–48 mm diam (⌀ = 39 mm); PDA 35–42 mm diam (⌀ = 36 mm). Colonies at 37 °C in 7 d: SAB 25–40 mm diam (⌀ = 34 mm); MEA 30–37 mm diam (⌀ = 32 mm); PDA 32–34 mm diam (⌀ = 33 mm).

Material examined: New Zealand, hedgehog (*Erinaceus europaeus*), M.J. Marples (ex-holotype culture CBS 511.73 = ATCC 28443 = IMI 101051 = NCPF 375). The Netherlands, Delft (Diagnostic Center SSDZ), arm skin, human, 1979 (CBS 344.79). United Kingdom, Bristol (General Hospital Bristol), human, 1972 (IHEM 19619 = RV 28925); ibid., culture IHEM 19621 = RV 28927.
Distribution and ecology: Trichophyton erinacei (Quaife 1966) is a zoophilic species that is common in wild-living and pet hedgehogs worldwide. The pathogen was originally described in the European hedgehog (*Erinaceus europaeus*), occurring naturally in the UK and Northern and Western Europe; it has also been imported to New Zealand and Japan (Morris and English 1969; Smith and Marples 1964; Takahashi et al. 2003). The African wildliving four-toed hedgehog (*Atelerix albiventris*) is another host of *T. erinacei*. The prevalence of the pathogen is high in both wild-living and pet hedgehogs, resulting in a significant increase in human infections due to *T. erinacei*, especially those contracted from pet hedgehogs, in recent years (Abarca et al. 2017; Hubka et al. 2018d; Kargl et al. 2018). The presentation in hedgehog range from asymptomatic infection (Fig. 22) to extensive involvement of the body surface. The infection is predominantly located on the head and usually spread slowly (Morris and English 1973; Schauder et al. 2007; Takahashi et al. 2002). In human, extremities are affected in cca 70–80% of reported cases (Fig. 22), although tinea corporis, barbae, faciei (Fig. 22), capitis and onychomycosis have been also reported (Concha et al. 2012; English et al. 1962; PiérardFranchimont et al. 2008).

Notes: The morphology of *T. erinacei* resembles that of *T. africanum* and *T. mentagrophytes*. Compared to *T. mentagrophytes*, with a dark colony reverse colour, the colony reverse colour of *T. erinacei* is pale. These species also differ in the production of spiral hyphae, which are absent in *T. erinacei*, and by the general shape of microconidia, which are mostly globose or subglobose in *T. mentagrophytes*. The species is strongly associated with hedgehogs, and identification is thus only difficult when isolated from infected humans with incomplete anamnestic data. The closely related taxa *T. eriotrephon* and *T. verrucosum* are easily distinguishable from *T. erinacei* by their slower growth rates (Fig. 11) and relatively poor sporulation (sporulation usually absent in *T. verrucosum*). Additionally, *T. eriotrephon* can be differentiated from *T. erinacei* by the production of an intense

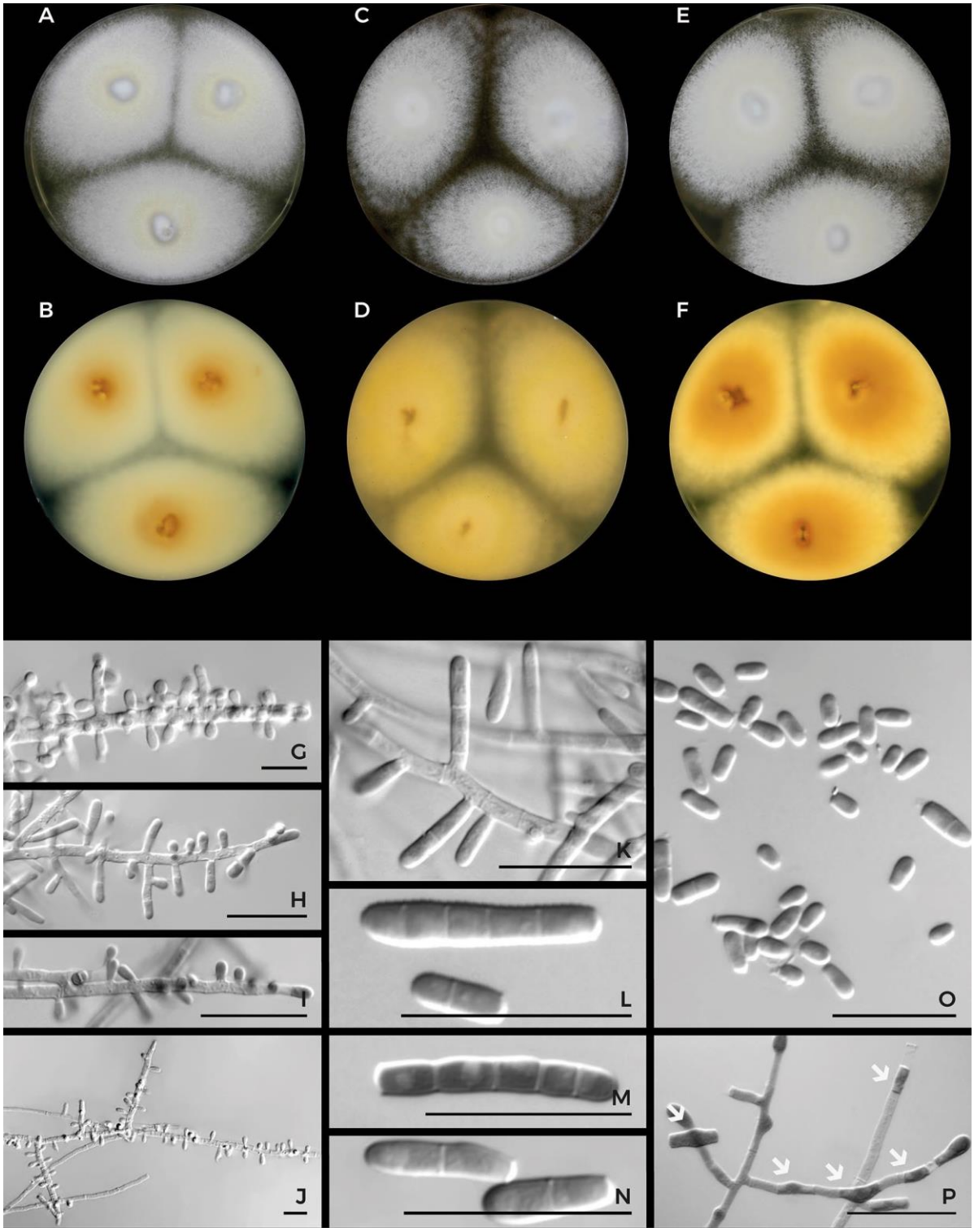


Fig. 21 Macromorphology and micromorphology of *Trichophyton erinacei*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**a**, **b**), malt extract agar (**c**, **d**) and potato dextrose agar (**e**, **f**). Conidiophores bearing microconidia (**g**–**j**) and macroconidia (intermediate forms) (**k**); macroconidia (**l**–**n**); free microconidia and macroconidia (two-celled intermediate forms) (**o**); intercalary conidia—marked with arrows (**p**). Scale bars = 20 μm

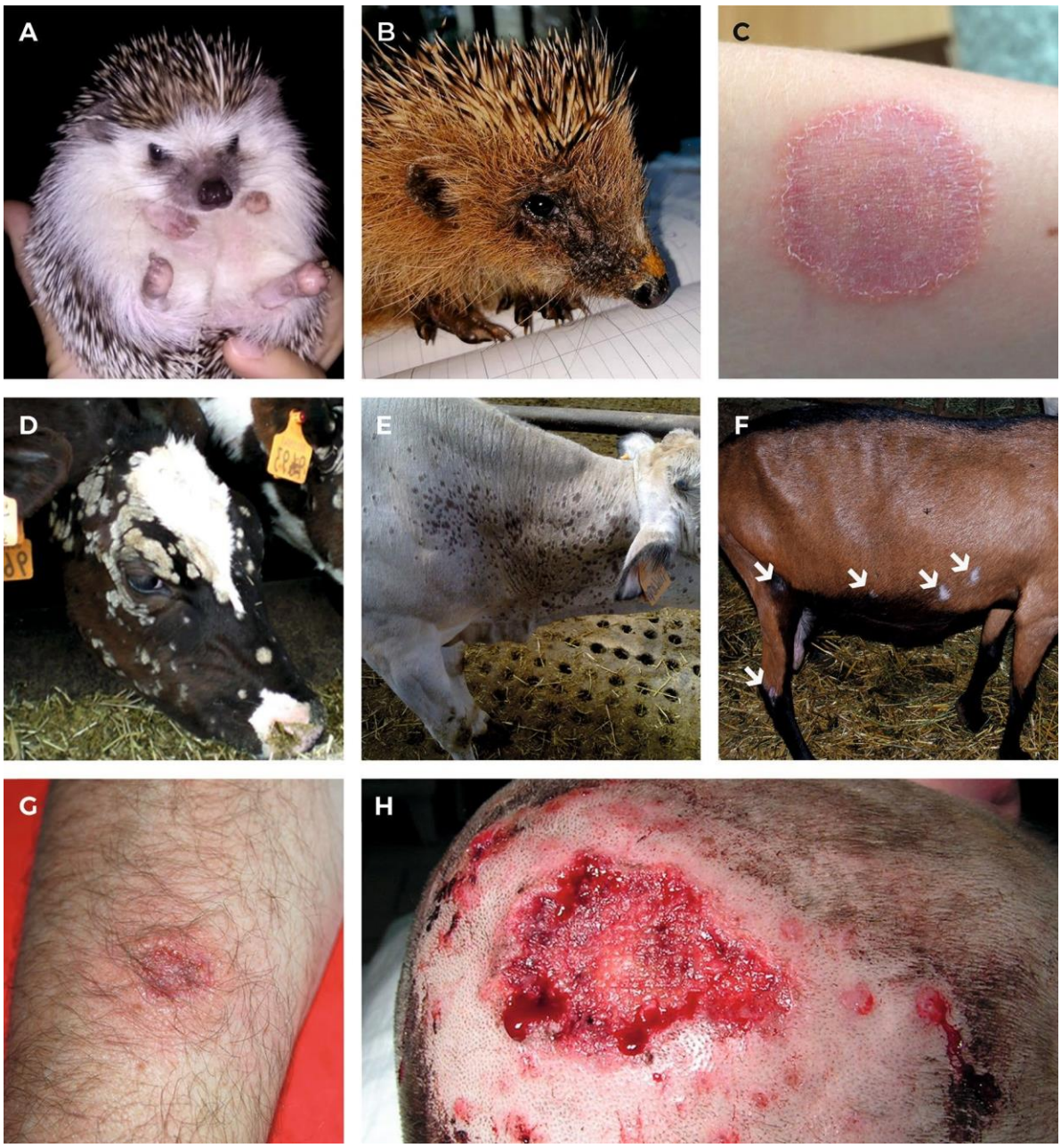


Fig. 22 Clinical presentation of infections caused by *Trichophyton erinacei* clade species in animals and humans. *Trichophyton erinacei*: four-toed hedgehog (*Atelerix albiventris*) without apparent clinical signs of infection (**a**), a source of tinea corporis infection in a pet breeder (Lysková et al. , 2018); wild European hedgehog (*Erinaceus europaeus*) with facial skin lesion (**b**), image courtesy of Veronica Risco-Castillo; tinea corporis on the left forearm caused by *T. erinacei* (**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**)

reddish-brown pigment and microconidia with variable shapes. Only the MAT1-1–2 idiomorph was detected among the *T. erinacei* isolates examined here.

Trichophyton eriotrephon Papegaay, Ned. Tijdschr. Geneesk. 69: 885. 1925—Fig. 23

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

Culture characteristics (7 days at 25 °C): Colonies on SAB 17–25 mm diam (⌀ = 21 mm), white (#F5F5F0) or light yellowish brown (#E3D6A1), flat with radially wrinkled centre, velvety to delicately granular, edge entire, reverse deep reddish brown (#882D17), diffuse pigment strong reddish brown (#6E2615) produced into the medium (less intense in IHEM 24340). Colonies on MEA 17–25 mm diam (⌀ = 21 mm), white (#F5F5F0) to pale yellow (#C2B280) in the centre, flat with or without radially wrinkled centre, velvety to delicately granular, edge submerged and filliform, reverse vivid red (#841B2D) to deep reddish brown (#882D17) (yellow reverse in IHEM 24340). Colonies on PDA 17–24 mm diam (⌀ = 22 mm), white (#F5F5F0) to light yellowish brown (#E3D6A1) in the centre, flat or umbonate, with raised centre (radially wrinkled in CBS 220.25), velvety or downy, edge submerged to filliform, reverse vivid orange (#F38400) to strong yellowish brown (#80461B) in the centre. Colonies at 30 °C in 7 d: SAB 22–32 mm diam (⌀ = 27 mm); MEA 28–31 mm diam (⌀ = 31 mm); PDA 22–25 mm diam (⌀ = 24 mm). Colonies at 37 °C in 7 d: SAB 0–3 mm diam (⌀ = 1 mm); no growth on MEA and PDA.

Material examined: The Netherlands, human dermatophytosis, 1925, J. Papegaay (ex-type culture CBS 220.25). Belgium, Marke, dog skin and hair (Jack Russell terrier), 2010 (IHEM 24340).

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

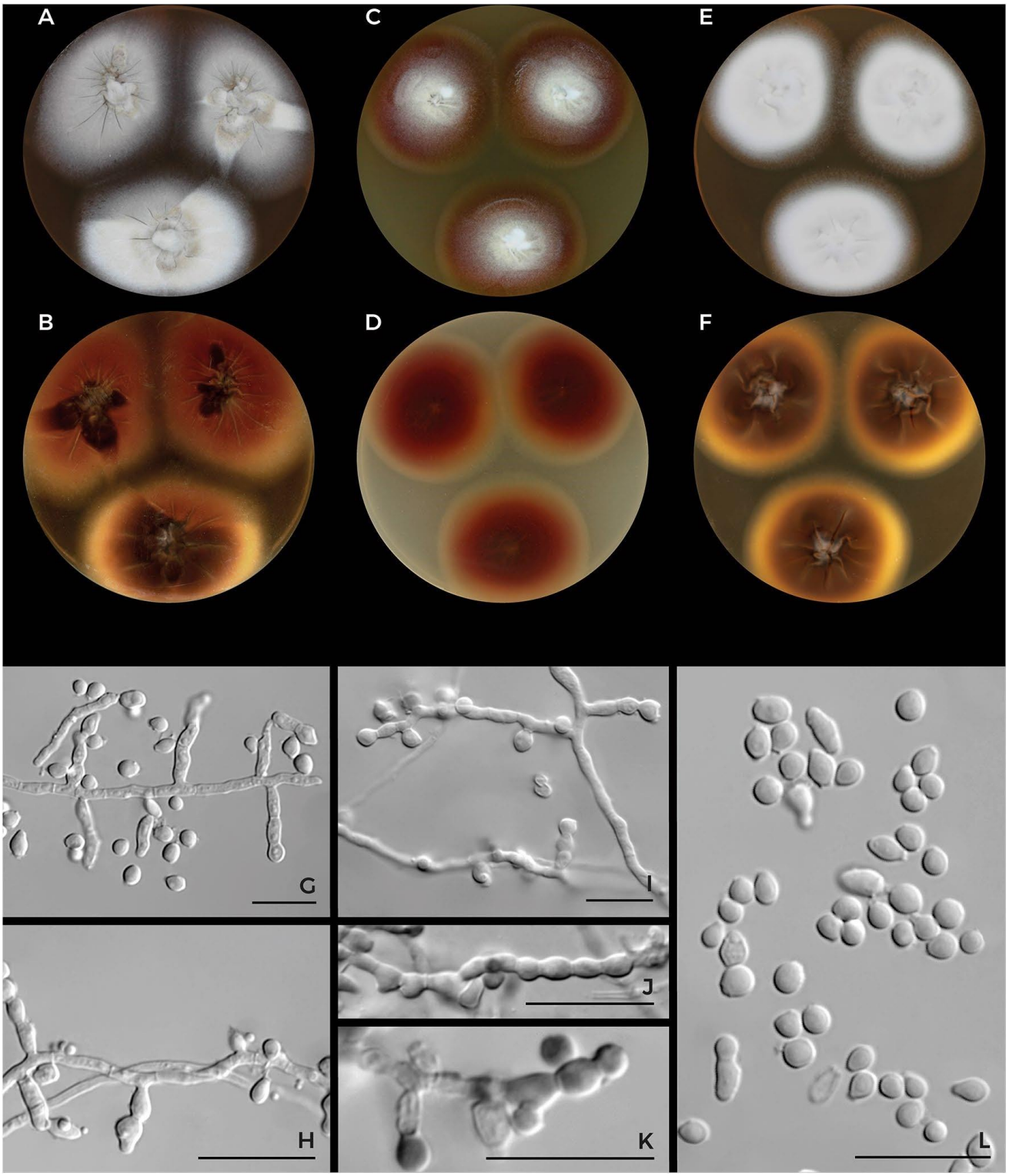


Fig. 23 Macromorphology and micromorphology of *Trichophyton eriotrephon*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**a**, **b**), malt extract agar (**c**, **d**) and potato dextrose agar (**e**, **f**). Conidiophores bearing microconidia and intercalary conidia (**g**–**k**); microconidia and intercalary conidia with variable shape (**l**). Scale bars = 20 µm

Notes: The morphology of *T. eriotrephon* only slightly resembles species from the *T. benhamiae* clade in its redbrown colony reverse colour. The conidiophores of *T. eriotrephon* are mostly loose and poorly branched compared to those of zoophilic species from the *T. benhamiae* clade, with grape-like conidiophores. Other typical characteristics include the production of a diffuse red-brown pigment on SAB, microconidia with variable shapes and the absence of macroconidia. These characteristics, together with the absence of or restricted growth at 37 °C, differentiate *T. eriotrephon* from all other species of the *T. benhamiae* complex. The MAT1-1-1 idiomorph of the mating type gene was detected in both *T. eriotrephon* isolates examined here.

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

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

Culture characteristics (7 days at 25 °C): Colonies on SAB 18–22 mm diam (⌀ = 20 mm), white (#F5F5F0) to pale orange yellow (#FFF587) or light orange yellow (#FAD6A5), flat, raised and furrowed, or cerebriform, velvety to slightly powdered, edge entire, lobate, or submerse, reverse light orange yellow (#F8DE7E) to deep orange yellow (#C9AE5D), dark brown in some strains. Colonies on MEA 5–18 mm diam (⌀ = 13 mm), white (#F5F5F0) to pale orange yellow (#F3E5AB), raised in the centre, frequently wrinkled, velvety or waxy, edge entire, lobate, or submerse, reverse light orange yellow (#F8DE7E) to vivid orange yellow (#F6A600), dark brown in some strains. Colonies on PDA 8–18 mm diam (⌀ = 14 mm), white (#F5F5F0) to pale orange yellow (#F3E5AB), flat or with raised centre, velvety or waxy, edge entire, lobate, or submerse, reverse light orange yellow (#F8DE7E), dark brown in some strains. Colonies at 30 °C in 7 d: SAB 10–23 mm diam (⌀ = 18 mm); MEA 8–10 mm diam (⌀ = 9 mm); PDA 9–10 mm diam (⌀ = 9 mm). Colonies at 37 °C in 7 d: SAB 9–15 mm diam (⌀ = 10 mm); MEA 11–12 mm diam (⌀ = 11 mm); PDA 12–14 mm diam (⌀ = 13 mm).

Material examined: Unknown locality, cow, 1953, F. Blank (ex-neotype culture CBS 365.53). Czechia, Pardubice, dermatophytosis in 21-year-old woman (contact with cattle), 2011, K. Mencl (CCF 4612). Czechia, Hlinsko, dermatophytosis in 58-year-old woman (contact with cattle), 2011, K. Mencl (CCF 4613). Czechia, Tábor, dermatophytosis in 38-year-old woman (contact with cattle), 2014, N. Mallátová (CCF 4889).

Distribution and ecology: Trichopyhton verrusocum is a zoophilic species typically found in cattle and other ruminants (Fig. 22), but it can easily spread to humans and animals, including horses, donkeys, camels, rabbits, dogs, cats, pigs, and even birds (Ali-Shtayeh et al. 1988; Dvořák et al. 1965; Georg 1960; Chermette et al. 2008; Khosravi and Mahmoudi 2003). The species is distributed worldwide, but the incidence of infections in cattle and man has been decreased in many regions by specific preventive measures, especially by vaccination programmes or changes in agricultural systems, such as reduction of the number of cattle in breeding units, and infections in humans have decreased proportionally (Lund et al. 2014; Seebacher et al. 2008). Human patients usually develop aggressive inflammatory skin lesions usually located on extremities and head (Fig. 22), which may be accompanied by constitutional symptoms, such as fever and lymphadenopathy (Courtellemont et al. 2017; Silver et al. 2008). Tinea barbae and capitis are relatively common clinical forms which can result in irreversible scarring and alopecia.

Notes: The morphology of *T. verrucosum* resembles that of *T. bullosum* and *T. concentricum*. For distinguishing characters see *T. bullosum* description. Only MAT1-2-1 idiomorph was detected in all strains examined here and in all strains analyzed by other reserchers (Kano et al. 2014; Kosanke et al. 2018).

***Trichophyton bullosum* clade**

Trichophyton africanum Cmokova & Hubka, ***sp. nov.***—
MycoBank MB835890; Fig. 25

Etymology: Refers to the origin of the ex-type strain.
Typus: MOZAMBIQUE, human, 1969, M.J. CamposMagalhaes, PRM 944418 (holotype); ex-holotype culture IHEM 4032 (= ATCC 28064 = RV 25293 = CM 3440 = CCF 6493).

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

Culture characteristics (7 days at 25 °C): Colonies on SAB 30–32 mm diam (⌀ = 31 mm), white (#F2F3F4) to pale yellow green (#F2F3E5), granular, slightly raised in the center, margin diffuse, reverse light orange yellow (#FBC97F) in the marginal part, strong orange (#ED872D) in the center.

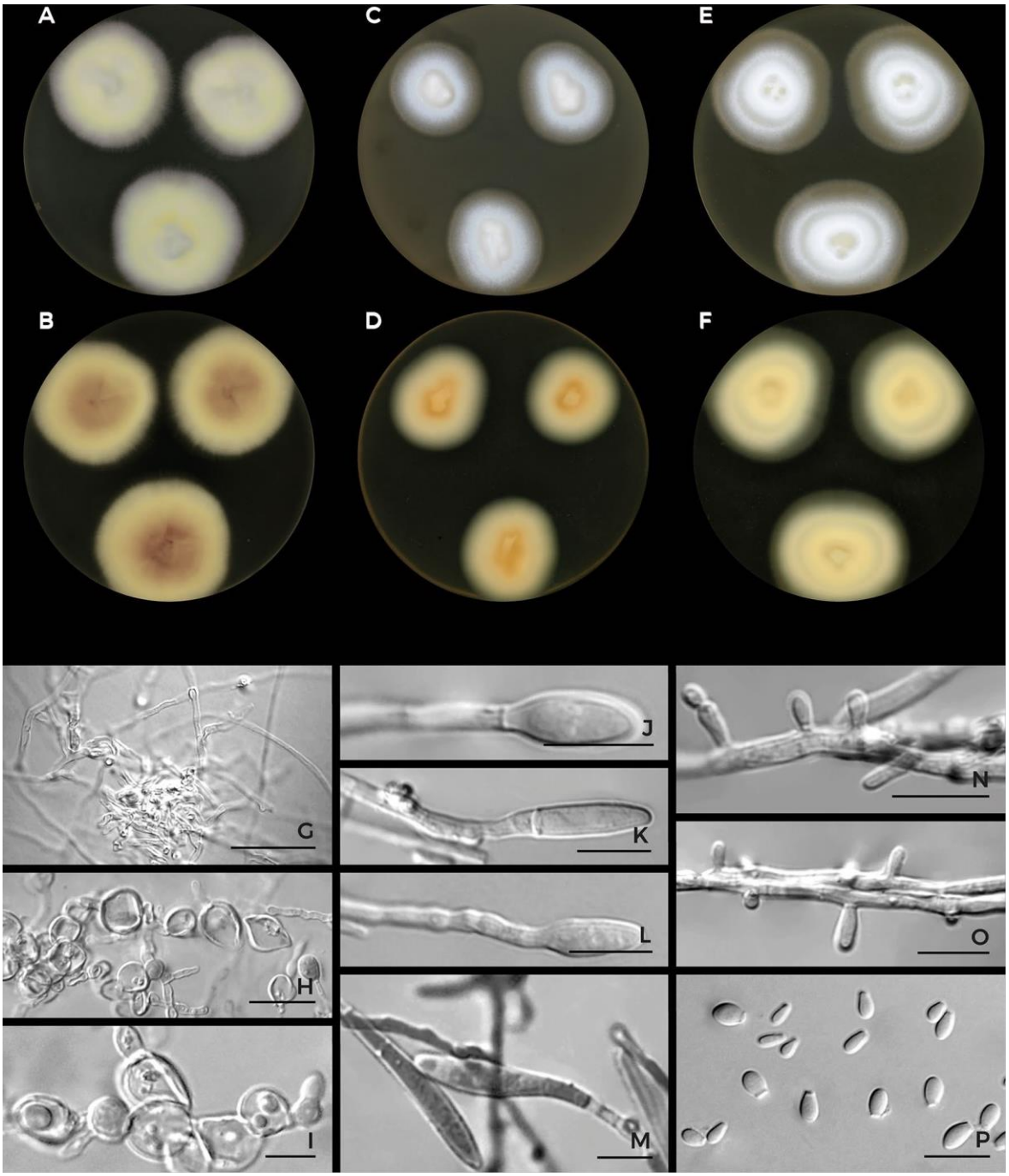


Fig. 24 Macromorphology and micromorphology of *Trichophyton verrucosum*. Colonies after three weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**a**, **b**), malt extract agar (**c**, **d**) and potato dextrose agar (**e**, **f**). Clumps of vegetative hyphae (**g**); chlamydospores in chains (**h**, **i**); macroconidia (**j**–**m**); conidiophores (fertile hyphae) with sessile microconidia (**n**, **o**); microconidia (**p**). Scale bars = 20 µm.

Colonies on MEA 28–35 mm diam (⌀ = 30 mm), white (#F2F3F4), granular, flat, margin entire, reverse uncoloured to light orange yellow (#FBC97F). Colonies on PDA 27–28 mm diam (⌀ = 28 mm), white (#F2F3F4) to pale yellow green (#F2F3E5), granular to floccose, slightly raised in the center, margin

entire, reverse pale yellow (#F3E5AB) to pale orange yellow (#FAD6A5) in the marginal part, dark orange yellow (#BE8A3D) in the center. Colonies at 30 °C in 7 d: SAB 40–45 mm diam (⌀ = 43 mm); MEA 35–45 mm diam (⌀ = 39 mm); PDA 35–40 mm diam (⌀ = 36 mm). Colonies at 37 °C in 7 d: SAB 21–24 mm diam (⌀ = 24 mm); MEA 20–29 mm diam (⌀ = 23 mm); PDA 20–22 mm diam (⌀ = 21 mm).

Material examined: Mozambique, human, 1969, M.J. Campos-Magalhaes (PRM 944418, holotype, dried culture); ex-holotype culture IHEM 4032 = ATCC 28064 = RV 25293 = CM 3440 = CCF 6493). Belgium, Bruges, human fingernail, 1978 (IHEM 19628 = RV 40614). South Africa, human skin, 1971, K. Scott (IHEM 4033 = ATCC 28065 = CBS 808.72 = CECT 2895 = NCPF 456 = RV 27926).

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

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

Trichophyton bullosum Lebasque, Les Champignons des Teignes du Cheval et des Bovidés: 53. 1933—Fig. 26

Vegetative hyphae smooth, septate, inflated, often branched and with knob-like terminations, hyaline 1.5–4 µm diam (mean ± sd; 2.7 ± 0.7). *Chlamydospores* abundant, spherical, oval or irregular, occasionally in chains, 4–9(–20) µm in diam. *Microconidia* and *macroconidia* not observed in the isolates examined in this study, but they were observed by Lebasque (1933) under specific conditions. *Spiral hyphae* absent.

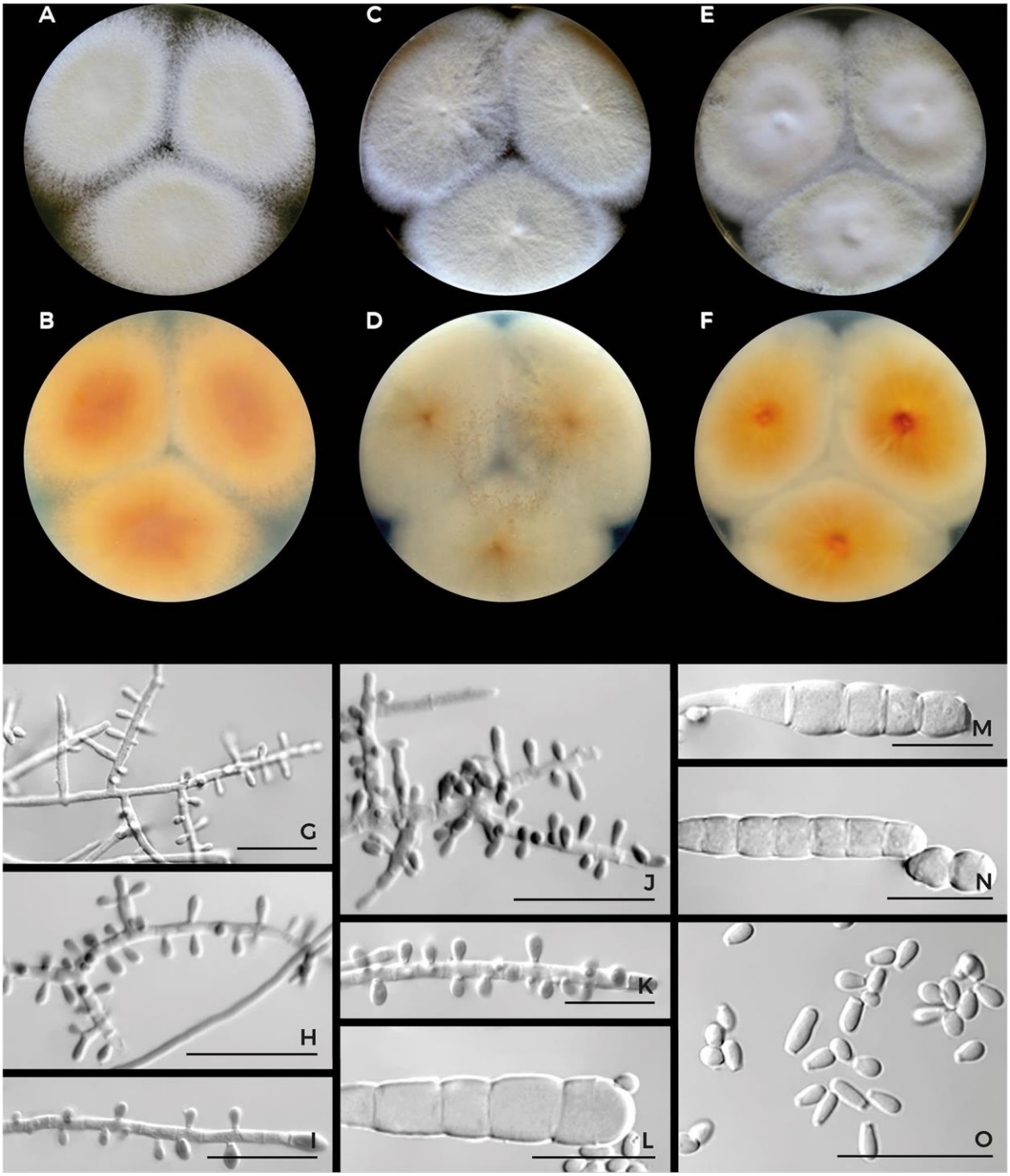


Fig. 25 Macromorphology and micromorphology of *Trichophyton africanum*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (a, b), malt extract agar (c, d) and potato dextrose agar (e, f). Conidiophores bearing microconidia (g–k); macroconidia (l–n); microconidia (o). Scale bars = 20 µm.

Culture characteristics (7 days at 25 °C): Colonies on SAB 11–12 mm diam (⌀ = 12 mm), white (#F2F3F4) to pale yellowish pink (#ECD5C5) or pale orange yellow (#FAD6A5), umbonate, radially furrowed, membranous or slightly velvety, edge submerged or filiform, reverse light yellow (#F8DE7E). Colonies on MEA 8–12 mm diam (⌀ = 10 mm), White (#F2F3F4), pale yellow (#F3E5AB) or vivid

orange yellow (#F6A600), flat with raised and cerebriform center, membranous, edge entire or submerged with dendritic growth, reverse light yellow (#F8DE7E). Colonies on PDA 7–9 mm v diam (⌀ = 8 mm), white (#F2F3F4), pale yellowish pink (#ECD5C5) or pale orange yellow (#FAD6A5), circular, flat, umbonate, membranous, edge entire, reverse light yellow (#F8DE7E). Colonies at 30 °C in 7 d: SAB 11–14 mm diam (⌀ = 13 mm); MEA 11–12 mm diam (⌀ = 11 mm); PDA 12–13 mm diam (⌀ = 12 mm). Colonies at 37 °C in 7 d: SAB 8–10 mm diam (⌀ = 9 mm); MEA 9–10 mm diam (⌀ = 10 mm); PDA 9–10 mm diam (⌀ = 9 mm).

Material examined: France, horse, J. Lebasque (ex-type culture, CBS 363.35 = LP 770). Czechia, skin lesions in horse, 2013, P. Lysková (CCF 4831). Egypt, near Cairo, skin lesion in donkey (*Equus asinus*), 2015, A. Peano (CCF 5730).

Distribution and ecology: Trichophyton bullosum is a zoophilic species known from infections in donkeys and horses (Fig. 27). It is distributed in Europe, North Africa and the Middle East (Lebasque 1933; Lysková et al. 2015; Sabou et al. 2018; Sitterle et al. 2012). but produce abundant chlamydospores, frequently in the form of chains. All mentioned species are relatively strongly associated with their hosts and/or with a typical clinical manifestation (cattle ringworm caused by *T. verrucosum*; dermatophytosis caused by *T. bullosum* in horses and donkeys; tinea imbricata caused by *T. concentricum* in humans). Therefore, detailed anamnestic data can facilitate their identification. Molecular genetic methods may be necessary to verify the identification of some isolates. For the differentiation of *T. bullosum* from the most closely related species, *T. africanum*, see the description of *T. africanum*. Only the MAT1-1-1 idiomorph was detected among the *T. bullosum* isolates examined here.

Notes: Due to its slow grow rate, *T. bullosum* strongly resembles *T. verrucosum* and *T. concentricum*. These species either do not sporulate or sporulate poorly (especially on sugar-rich media such as SAB)

DISCUSSION

Species delimitation issues in *Trichophyton*

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

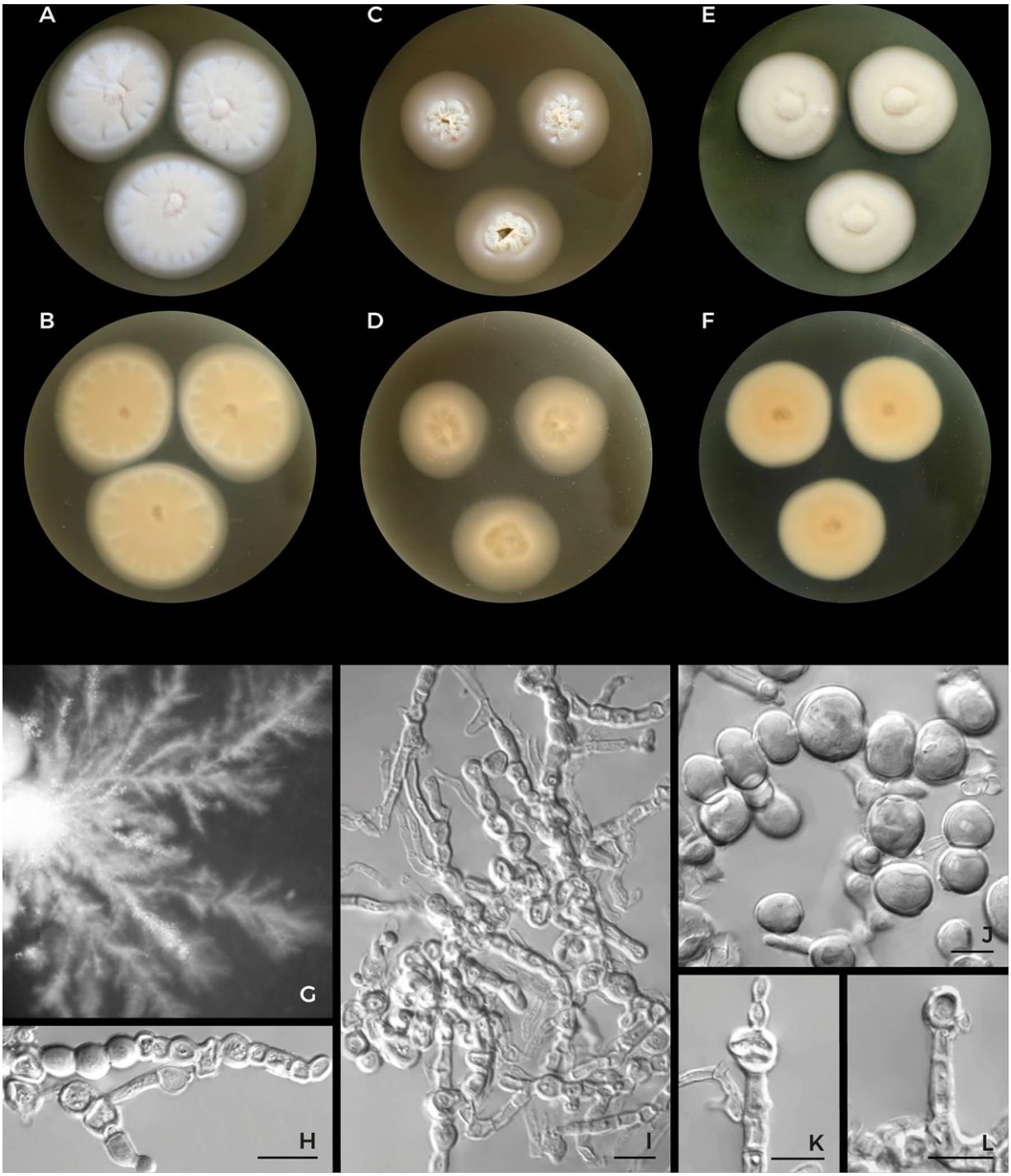


Fig. 26 Macromorphology and micromorphology of *Trichophyton bullosum*. Colonies after three weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**a**, **b**), malt extract agar (**c**, **d**) and potato dextrose agar (**e**, **f**). Detail of colony with submerged, dendritic growth on Sabouraud's dextrose agar supplemented with cycloheximide and chloramphenicol after 3 months of cultivation at 25 °C (**g**). Thickwalled vegetative hyphae with numerous intercalary or terminal chlamydospores (**h**, **i**); chlamydospores in chains and free chlamydospores (**j**), vegetative hyphae with terminal chlamydospores (**k**, **l**). Scale bars = 20 µm

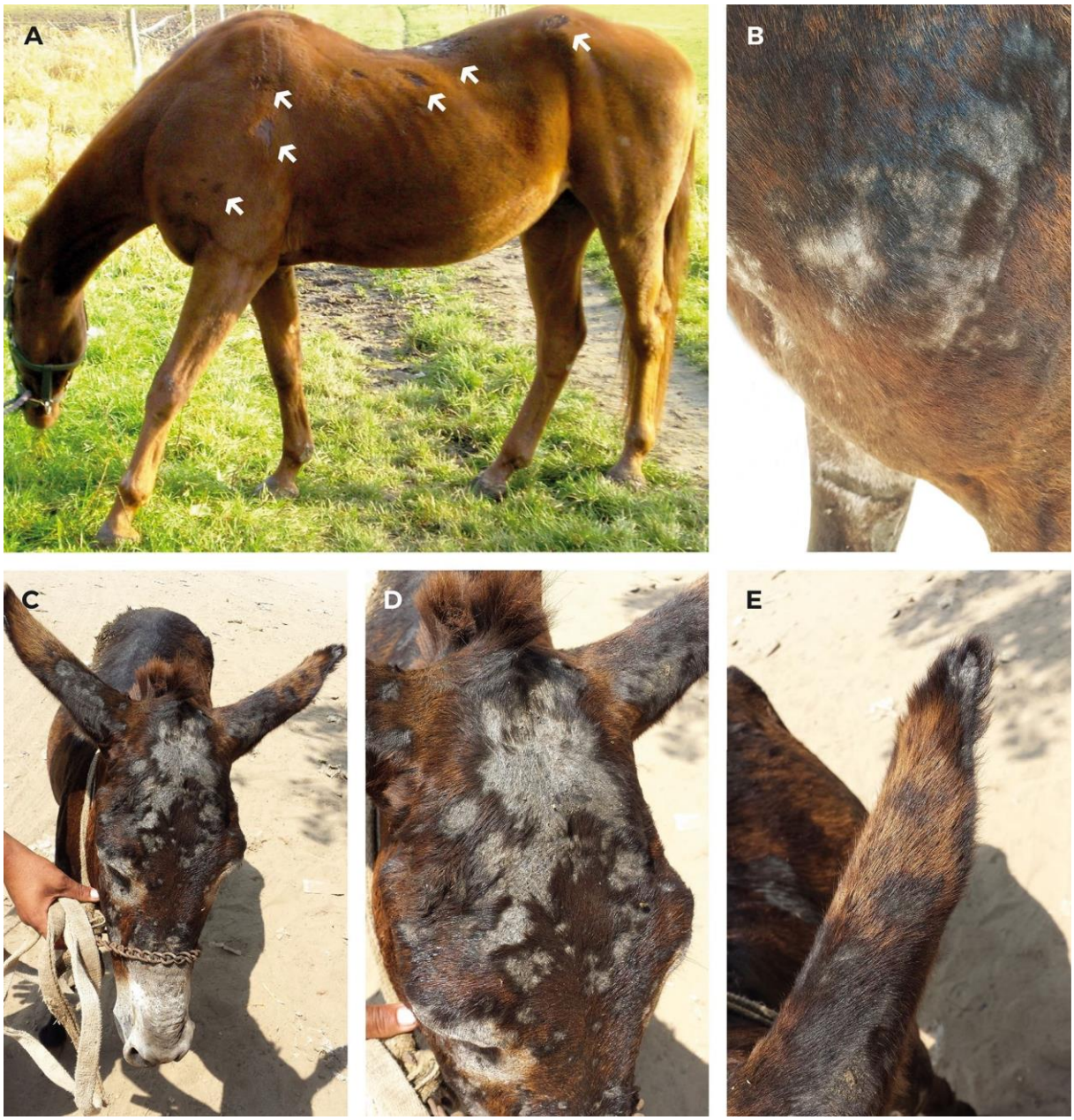


Fig. 27 Clinical presentation of infections caused by *Trichophyton bullosum*: patches of hair loss in the saddle area, shoulders, hip bones, withers and upper chest of a horse (**a**), isolate CCF 4831 Lysková et al. 2015); scaling patches of hair loss located on the head, chest and legs of a donkey (**b**–**e**), isolate CCF 5730

Phenotypic criteria are usually relatively effective in routine diagnostics for major dermatophyte species or species complexes. However, as in other fungi, we have found similarities between species or morphotypes across unrelated dermatophytes, resulting in misdiagnosis in practice (Lysková et al. 2015; Summerbell 2011; Uhrlaß et al. 2018). There is also considerable intraspecific phenotypic variability in

other species or species complexes that is not correlated with molecular taxonomy (Heidemann et al. 2010; Kandemir et al. 2020; Su et al. 2019). Moreover, the success rate of phenotypic identification frequently depends on the age of isolates because of the rapid degeneration of important portions of cultures (de Hoog et al. 2017). Consequently, it can be difficult to maintain and reproduce phenotypic characters over decades for the purposes of taxonomic studies.

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

The specific problems in species delimitation in *Trichophyton* can be demonstrated by the example of the *T. mentagrophytes* and *Trichophyton rubrum* complexes. It was generally assumed that the differentiation of zoophilic *T. equinum* (main host = horse) from closely related anthropophilic *T. tonsurans* would be possible based on the ecological preferences, nutritional requirements, and MAT gene idiomorphs (Summerbell et al. 2007; Woodgyer 2004). Kandemir et al. (2020) examined 67 isolates and found that none of the five selected phylogenetic markers were able to unambiguously separate these species (probably due to incomplete lineage sorting) according to differences in their MAT genes, ecology and nicotinic acid requirements. It is postulated that these species evolved very recently and that the speciation process might not yet be complete (Kandemir et al. 2020). Another taxonomically problematic species pair is *T. mentagrophytes*/*T. interdigitale*. According to the traditional concept promoted by de Hoog et al. (2017), *T. mentagrophytes* is a zoophilic species in which both MAT idiomorphs are present in the population, resulting in relatively high intraspecific genetic variability. By contrast, anthropophilic *T. interdigitale* is a clonal lineage (consisting only of the MAT1-1-1 idiomorph) that is almost exclusively associated with onychomycosis and tinea pedis. Although the correlation between the genotype and the clinical manifestation or source of isolates has been repeatedly demonstrated, the correlation between ITS genotype and phenotype is relatively poor (Dhib et al. 2017; Heidemann et al. 2010; Pchelin et al. 2016). Currently, the molecular diagnosis of these species is mostly based on several unique sites in the ITS region, and phylogenies usually resolve *T. mentagrophytes* as para- or polyphyletic with *T. interdigitale* (Hainsworth et al. 2020; Heidemann et al. 2010; Nenoff et al. 2019; Pchelin et al. 2019; Singh et al. 2019; Taghipour et al. 2019). Both species names remain in use,

due to the epidemiological consequences associated with different sources of infections in particular. The laboratory diagnosis of *T. mentagrophytes* and *T. interdigitale* and that of *T. equinum* and *T. tonsurans* are further complicated by inaccurate or even impossible species differentiation using MALDI-TOF MS (da Cunha et al. 2018; Dukik et al. 2018; Hedayati et al. 2019; Nenoff et al. 2013; Suh et al. 2018).

Very similar species delimitation issues complicate the taxonomy of the anthropophilic *T. rubrum* complex, encompassing clonal lineages showing differences in their distribution and the clinical manifestation of associated infections (de Hoog et al. 2017; Gräser et al. 2000). The majority of molecular studies relying on the variability in the ITS region and microsatellite markers have revealed some support for 2–4 lineages (i.e., *T. rubrum*, *T. violaceum* and/or *T. soudanense* and/or *T. yaoundei*), but the number of species and their boundaries are still under debate (Gräser et al. 2007; Packeu et al. 2020; Su et al. 2019). MALDI-TOF MS showed promising results in the differentiation of these species/lineages (Packeu et al. 2020). Detailed genomic, epigenetic and multigene phylogenetic studies on a large number of samples can resolve delimitation issues between these species in the future (Pchelin et al. 2019; Singh et al. 2019; Zhan et al. 2018).

SNP detection by whole-genome sequence typing can be used to infer the genetic relatedness of *Trichophyton* isolates. This approach will ultimately become one of the methods of choice in the future with decreasing costs (Hadrich and Ranque 2015). Currently, the sequencing of ITS rDNA and population genetic markers such as microsatellites (Gräser et al. 2007; Kaszubiak et al. 2004; Pasquetti et al. 2013) or mixed-marker approaches (Abdel-Rahman et al. 2010) offers higher discriminatory power in the species differentiation of primary pathogenic dermatophytes compared to MLST approaches based on the currently available loci.

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

In this study, we encountered similar problems to those mentioned in the previous section in the *T. mentagrophytes* and *T. rubrum* complexes. However, a polyphasic approach combining independent molecular genetic markers (four DNA loci and 10 microsatellite loci) with phenotypic features and ecological data helped to overcome the majority of obstacles to species delimitation. Selected characteristics are schematically summarized in Fig. 28. Various forms of polyphasic approaches integrating different types of data, including morphological, physiological, exometabolite, ecological and molecular are increasingly used in many fungal order, e.g. Eurotiales, Pleosporales and Xylariales (Bhunjun et al. 2020; Houbraken et al. 2020; Kuhnert et al. 2017; Lambert et al. 2019; Samarakoon et al. 2020). These complex species delimitation approaches are basis for stable taxonomy that is less prone to errors compared to less robust approaches. Molecular data from gradually increasing number of nonlinked loci become a regular part of these approaches which prevents the impact of paralogous genes,

incomplete lineage sorting, non-reciprocal monophyly and other phenomena on taxonomic conclusions (Hubka and Kolařík 2012; Matute and Sepúlveda 2019; Stadler et al. 2020; Steenkamp et al. 2018).

In this study, we showed that isolates that were designated in the past as the Americano-European race of *T. benhamiae* harbour five taxa (three species and two varieties). The strains with the so-called white phenotype do not represent monophyletic entities and correspond to *T. benhamiae* var. *benhamiae*, *T. japonicum* and *T. europaeum*, while the yellow phenotype strains correspond to *T. benhamiae* var. *luteum*. Isolates of the African race of *T. benhamiae* referred to as *T. africanum* herein are phylogenetically distant and are most closely related to *T. bullosum*.

None of the four *sequence markers* alone was able to unequivocally differentiate all species within the *T. benhamiae* complex and provide accurate identification in 100% of cases. The ITS region contained a diagnostic position for all nine species, but the differentiation of *T. europaeum* and *T. japonicum* relied on a single substitution (Table S11). In addition, the identification of isolate IHEM 25139, with a probable hybrid origin, failed as described above. The *gapdh* gene was useful for differentiation between *T. europaeum* and *T. japonicum,* but some pairs of sister species shared identical sequences (i.e., *T. benhamiae* and *T. concentricum, T. verrucosum* and *T. eriotrephon*) (Table S11). The *tef1-α* gene differentiated all species except for *T. europaeum* and *T. japonicum* (Table S11). The *tubb* gene presented the least discriminatory power and failed to differentiate species within the *T. benhamiae* clade but could be used for species identification in the *T. erinacei* and *T. bullosum* clades. Insufficient discriminatory power of the *tubb* gene has been reported in many other *Trichophyton* species (Kandemir et al. 2020; Packeu et al. 2020; Suh et al. 2018). The unique substitutions observed within the DNA loci of the *T. benhamiae* clade species will be the basis for reliable species identification in practice (Table S11). The taxonomic significance of these unique sites is unambiguous, as they correspond to independent microsatellite markers and phenotypic and ecological data, indicating the reproductive isolation of recognized taxa.

While sequence markers were shown to be useful for the diagnosis of *T. benhamiae* complex species, they were not able to distinguish the two varieties of *T. benhamiae*. The only intraspecific variation was a single substitution in the *tef1-α* gene. This substitution was able to differentiate all *T. benhamiae* var. *luteum* isolates from the majority of *T. benhamiae* var. *benhamiae* strains, with the exception of two isolates from cluster C2, probably due to incomplete lineage sorting between these recently diverged lineages. The results of other analyses clearly indicated that *T. benhamiae* var. *luteum* is an emerging entity that is distinct both qualitatively (at the population genetic level and according to phenotypic differences) and ecologically (showing different hosts and distributions). The differentiation of this taxon has clinical relevance, due to which we decided to reassign it as variety of the nearest recombining ancestor, *T. benhamiae* var. *benhamiae*. We chose this conservative approach rather than the proposals of a new species because of the impossibility of distinguishing this entity using available DNA sequence markers, as is the current standard in fungal taxonomy.

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

Phenotypic and ecological data added another important piece to the taxonomic puzzle. *Trichophyton benhamiae* var. *luteum* can be identified by its slow growth on all media at all temperatures and its uniform phenotype (yellow reverse side of colonies and absence of macroconidia; all strains exhibit only mating type MAT1-1-1). The closely related *T. benhamiae* var. *benhamiae* is only found in the USA (mostly dogs) and exhibits strikingly different colonies with a brown to red-brown reverse side, macroconidium production and larger microconidia than *T. benhamiae* var. *luteum*. This variety shows the most rapid growth among the species from the *T. benhamiae* clade; isolates with both MAT gene idiomorphs were detected among the examined strains. *Trichophyton europaeum* is the second most common species from the *T. benhamiae* complex occurring in Europe and is responsible for human and guinea pig infections. While *T. japonicum* is currently responsible for the majority of human and animal (rabbits and guinea pigs) infections in Japan, it also occurs in Europe at low frequencies. Reliable differentiation of these species is only possible by molecular methods. *Trichophyton japonicum* and *T. europaeum* differ strikingly in the distribution of mating type genes in their populations. Detailed distinguishing characteristics of particular species are listed in the Taxonomy section, and proposed identification procedure in clinical practice is illustrated in Fig. 29.

Speciation through the geographic expansion, host jump and extinction of opposite mating type partners

The assessment of species boundaries via mating experiments (revealing biological compatibility) played an important role in the delimitation of many early species and the discovery of their sexual states. This approach based in principle on the biological species concept (BSC) is generally highly applicable in geophilic dermatophytes (Dawson and Gentles 1962; Hubka et al. 2015; Choi et al. 2012; Padhye and Carmichael 1972; Stockdale 1964). By contrast, the results of biological compatibility assessment can considerably disagree with the concept of classical species of anthropophilic and zoophilic dermatophytes. These species are evolutionarily young, and their phylogenetic divergence preceded the development of reproductive barriers, as demonstrated by interspecific hybrid induction

Fig. 28 Overview of selected data on ecology, phenotype and population genetics plotted on the simplified four-gene phylogeny of the *Trichophyton benhamiae* species complex. The icons of the hosts are explained in Fig. S1. Explanation of superscript numbers: [1]macroconidia observed by some authors (Rippon 1988; Pihet et al. 2008), [2]macroconidia were observed by Lebasque (1933) under specific conditions

between various primary pathogenic *Trichophyton* species in vitro (Anzawa et al. 2010; Kawasaki 2011; Kawasaki et al. 2009, 2010). However, it is highly unlikely that this kind of hybridization occurs naturally due to the different ecological niches of species, and the results of in vitro mating assays therefore cannot be extrapolated to a natural scenario. Additionally, the ratio of mating-type gene idiomorph is usually extremely imbalanced or one idiomorph is missing in the majority of anthropo- and zoophilic dermatophytes (Kosanke et al. 2018; Metin and Heitman 2017). This fact further limits or even prevents the possibility of using BSCs in the delimitation of these species. A similar phenomenon was observed by our group in almost all species from the *T. benhamiae* complex (Fig. 28), suggesting that the loss of opposite mating-type partners was an important driver of their evolution. The ancestors of many currently recognized pathogenic dermatophytes were likely sexually reproducing geophilic species and zoophilic species on free-living mammals (sexually reproducing, e.g., in soil surrounding burrows) with balanced ratios of opposite mating type individuals (Gräser et al. 2006; Summerbell 2011). Introduction to new areas and/or adaptation to a new host is probably a unique event in the evolution of many anthropo- and zoophilic dermatophytes, resulting in the extinction of one mating partner in the whole population of these species. Only some "clonal" offshoots of ancestral sexual dermatophytes probably maintain ongoing populations and follow independent evolutionary trajectories towards speciation (Gräser et al. 2006). Recent outbreak of *T. benhamiae* var. *luteum* follows the scenario reported in vertebrate pathogens *Batrachochytrium dendrobatidis* (James et al. 2009) and *Pseudogymnoascus destructans* (Trivedi et al. 2017). In that fungi, a single clone of one mating type migrated to new areas, meeting a naive host, what resulted in the high virulence, epidemic spread and formation of a clonal population. Alternatively, the extinction of one MAT gene in a population of dermatophytes may be caused by the preferential spread of strain(s) exhibiting an advantageous combination of alleles associated with higher virulence/transmission potential. Such a successful genotype may be significantly dominant in conditions with almost exclusive asexual transmission and may displace other genotypes. Such a situation is very likely to lead to an imbalance in the MAT gene ratio or even the loss of one MAT gene in the population. The extinction of strains belonging to one mating type is, for instance, observed in some populations of *M. canis* (Sharma et al. 2007), and different levels of virulence linked with mating-type idiomorphs have been repeatedly documented in fungal pathogens (Chang et al. 2000; Cheema and Christians 2011; Yue et al. 1999).

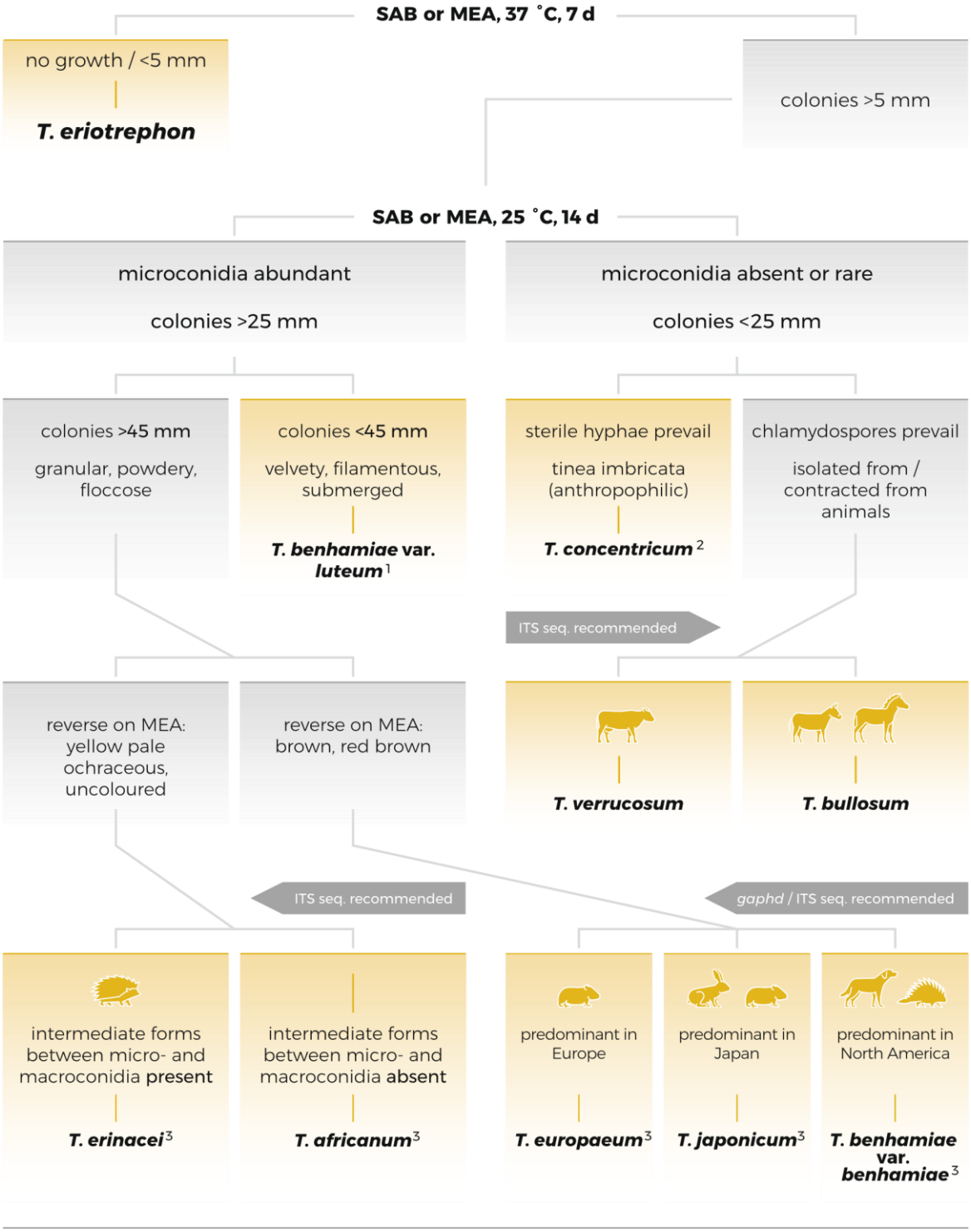
In the *T. benhamiae* clade, clonal reproduction is the dominant mode of dissemination (Dg, H, DW indices), and recombination is rare or absent in almost all populations according to the I_A. Despite the fact that only MAT12-1 idiomorph strains were present within *T. europaeum* strains, the null hypothesis of random mating was not rejected (Table S6, Fig. 9), suggesting the existence of recent recombination events in this species. As *T. japonicum* and *T. europaeum* consist of a single mating type and no recent recombination or gene flow has occurred between them, they should be conceptualized as separate, albeit clonal species, despite potential in vitro interbreeding (Gräser et al. 2006; Summerbell 2002). The disruption of gene flow between *T. benhamiae* clade species was reflected in high number of fixed alleles

(F_{ST} or G_{ST} indexes) (Table S5, Table S6) indicating reproductive isolation despite overlapping hosts (e.g. guinea pigs) and geographic distributions. This could be caused by pre- or postzygotic reproductive barriers, or absence of terrestrial reservoir for sexual reproduction.

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

The only exception among the examined isolates was strain IHEM 25139, isolated in 1963 by M. Takashio from guinea pig in France. This strain, identified here as *T. europaeum* based on the *gapdh* gene, shared some microsatellite alleles with *T. japonicum*. It also presented the MAT1-1-1 idiomorph of the MAT gene, typical of *T. japonicum* or *T. benhamiae* var. *benhamiae* cluster C3, and an atypical ITS1 region sequence with six substitutions compared to other *T. europaeum* strains, some of which are at positions crucial for the differentiation of *T. benhamiae* clade species (Fig. S2). It is possible that this strain originated from hybridization between *T. europaeum* and *T. japonicum*. The ecological niches of these species partially overlap, as they both occur in guinea pigs and some other animals that are frequently maintained together In addition, the coinfection of guinea pigs with two species or morphotypes has been repeatedly documented (Bartosch et al. 2019; Kupsch et al. 2017). This may suggest that the origin of *T. benhamiae* var. *luteum* lies in North America and that one or a few strains were recently introduced to Europe.

In such cases, the exchange of genetic information may likely occur not only through hybridization during saprophytic growth outside the host (possibly followed by introgressive hybridization) but also during coinfection of the same host through a parasexual cycle (anastomosis of hyphae, mitotic crossing-over and haploidization). Another strain with an ITS sequence identical to IHEM 25139 is IHEM 19622 (= RV 14389), which was not examined by our group (GenBank MK298816). These two strains with identical provenance were noted by Takashio to be atypical compared to other examined *A. benhamiae* isolates because of the less compact texture of their colonies (Takashio 1974)..



[1] dif. dg. *Trichophyton schoenleinii* (cause of favus, favic chandeliers)
[2] dif. dg. *Microsporum canis* (fast growth, spindle-shaped macroconidia, mostly cats and dogs)
[3] dif. dg. *T. mentagrophytes* (microconidia predominantly (sub)globose , spiral hyphae common , reverse frequently dark , host spectrum)

Fig. 29 Suggested procedure for *Trichophyton benhamiae* complex species identification in clinical practice based on phenotypic features (MEA and SAB; 25 and 37 °C) and molecular methods (if necessary). The icons of the hosts are explained in Fig. S1

These strains represent unique material for studying natural hybridization in dermatophytes. Their origin and genomic arrangement remain to be elucidated by genomic studies. The absence of these genotypes among the more recently isolated strains examined here and by others (no additional occurrence in GenBank) suggests that they were replaced by more successful genotypes.

Geographical distribution of *T. benhamiae* clade species

To understand the global distribution of the newly reassigned species in the *T. benhamiae* clade, we analysed 255 ITS rDNA sequences deposited in GenBank. The analysis enabled the identification of these records to the species level based on the species-specific substitutions in the ITS region (Table S10). This fact further supported the feasibility of the novel taxonomic classification proposed here. The ecological data resulting from the analysis were used as a basis for mapping the distribution of *T. benhamiae* clade species (Fig. 30; Table S10). The main limitations are the unavailability of epidemiological and DNA data from America, many Asian countries and Africa. As a result, the majority of analysed ITS sequences are from European countries and Japan, where dermatophyte research has a long tradition, and DNA-based identification is more commonly used. Additionally, it is not possible to distinguish two varieties of *T. benhamiae* based on the ITS region, but macro- and micromorphological characters described in some studies enable clear distinction of the varieties; the variety characteristics described below refer to such cases.

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

Based on current knowledge, white-phenotype strains (*T. europaeum* and *T. japonicum*) occurred in Europe before epidemic spread of yellow phenotype strains (*T. benhamiae* var. *luteum*). The oldest European strains representing *T. japonicum* are IHEM 4030 (collected before 1988 in Belgium) and IHEM 17701 (collected before 1997 in Spain). The oldest strains representing *T. europaeum* were collected before 1988 in Finland (Aho 1980) (Table S10) and more recently from Switzerland, in 2002 (IHEM 20159, IHEM 20161, IHEM 20162, IHEM 20163). The identity of other old white-phenotype strains reported in various European countries from the 1960s to 2000 (Fig. 1) is unclear due to the unavailability of isolates and/or sequence data. Both *T. japonicum* and *T. europaeum* were subsequently detected in Japan and some other countries (Fig. 30, Table S10). Outbreaks of infections caused by *T. benhamiae* var. *luteum* now seem to be limited to Europe, but an increasing number of infections can be

expected in non-European countries due to its recent introduction to other continents (de Freitas et al. 2019; Hiruma et al. 2015). Zoophilic *T. benhamiae* clade members have probably been brought into Japan with imported animals on several occasions and spread in Japan by the transportation of animals by breeders or pet shops, as suggested in a series of publications (Hiruma et al. 2015; Kano et al. 1998; Mochizuki et al. 2001; Takeda et al. 2012). The most prevalent species in Japan and South Korea (Jun et al. 2004; Lee et al. 2018, and pers. comm. with PL Sun) is *T. japonicum*. Other species are probably much less common: *T. europaeum* has been detected in guinea pig (unknown year of isolation) (Takeda et al. 2012), *T. benhamiae* var. *benhamiae* was imported to a Japanese zoo from Canada and the USA (in 2000 and 2002) with North American Porcupines (Takahashi et al. 2008), and *T. benhamiae* var. *luteum* was detected in 2012 in common degu (Hiruma et al. 2015).

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

Animal trade certainly plays an important role in the spread of zoonotic dermatophytes to new geographic areas. It also erases original geographic areas of a species distribution. Consequently, it is difficult to trace the origin of particular species. The current worldwide distribution and prevalence of infections caused by *T. benhamiae* clade members are poorly known due to insufficient overall surveillance of dermatophytosis supported by molecular-based identification. This problem pertains to both human and veterinary medicine. In addition, our knowledge of the ecology of these pathogens is mostly limited to domestic animals and pets, and little is known about potential wild-living hosts. Therefore, any hypothesis about the species origin is based on very incomplete data and needs to be refined by future research.

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

The emergence and rapid spread of *T. benhamiae* in Europe in the last decade and the recent detection of this species in many other countries has been one of the major public health events in the field of zoonotic superficial mycoses in recent years. This fact underscores the need for the One Health integrative approach and closer collaboration between the veterinary profession, dermatologists, epidemiologists and public health personnel (Bontems et al. 2020; Hubka et al. 2018d; Nenoff et al. 2014). Infected and frequently asymptomatic animals may act as a recurrent source of infections in other animals and humans. Interdisciplinary cooperation is needed to establish effective preventive measures for the control of infections. Genotyping techniques are often employed to gain insight into the dynamics of disease transmission, determine the source and routes of infections, confirm or rule out outbreaks, recognize virulent strains and regional and global changes in genotype patterns and evaluate the effectiveness of control measures (Ranjbar et al. 2014).

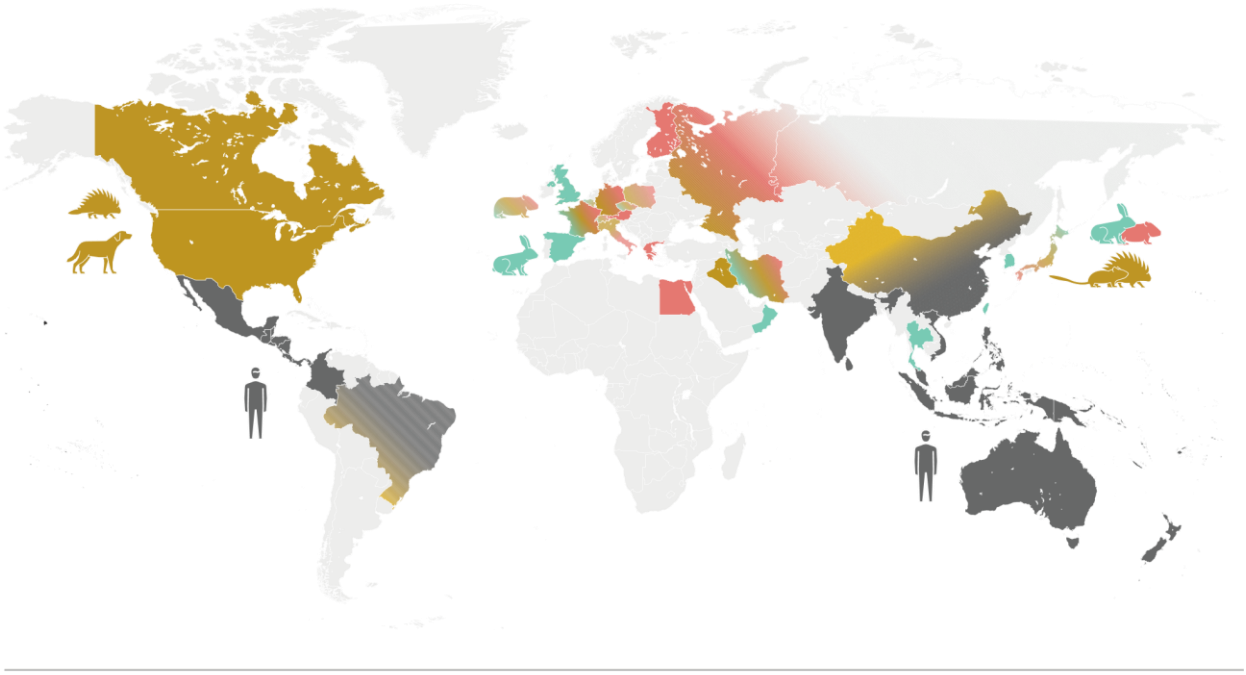


Fig. 30 Geographic distribution of species belonging to the *Trichophyton benhamiae* clade based on ITS rDNA available in GenBank database (Table S10). The main primary host(s) of species in different continents are marked by icons (explained in Fig. S1)

Other common issues in dermatophytes concern the differentiation of relapse versus reinfection and the determination of whether the infection is caused by one or more strains and if genotypes differ in their clinical manifestation. Many methods have been developed for the subtyping of dermatophytes, but a significant number of them are now obsolete, and their utility is frequently limited due to poor reproducibility or unsatisfactory strain differentiation (Abdel-Rahman 2008; Hubka et al. 2018d; Mochizuki et al. 2017). MLST typing approaches have been widely applied to many fungal pathogens (Bernhardt et al. 2013; Debourgogne et al. 2012; Maitte et al. 2013; Meyer et al. 2009; Prakash et al. 2016), but no such typing scheme has been evaluated and developed for dermatophytes, and the currently available loci usually lack sufficient discriminatory power to study the population structure of *Trichophyton* and *Microsporum* species in detail. Microsatellite markers are still among the most effective tools available for the subtyping of dermatophytes. Typing schemes have been developed for a limited number of species, including only *T. rubrum* (Gräser et al. 2007), *Nannizzia persicolor* (Sharma et al. 2008) and *M. canis* (Pasquetti et al. 2013; Sharma et al. 2007).

Polymorphisms in *T. benhamiae* (Americano-European race) were previously investigated by the RFLP analysis of the NTS region, which produced 11 different patterns in 46 isolates; this method successfully confirmed laboratory-acquired infections as well as familial outbreaks transmitted from pets (Mochizuki et al. 2002; Takeda et al. 2012). In this study, we developed a microsatellite typing scheme consisting of ten variable markers. This new typing scheme is currently the most powerful tool for the subtyping of

T. benhamiae clade species. It is easy to use and cost-effective due to its multiplex design. It is possible that the modified scheme can be used in other species in the *T. benhamiae* complex. Our preliminary data showed that at least 6 of 10 markers (CT21b, TAG16, TC20, TCA16, TC19, TC17a) are useful for the subtyping of another emerging pathogen, *T. erinacei*.

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

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

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Data availability The important fungal isolate used for experiments are publically available in the internationally recognized culture collections; newly generated DNA sequences are available in European Nucleotide Archive (ENA) database; alignments are available in the Supplementary material.

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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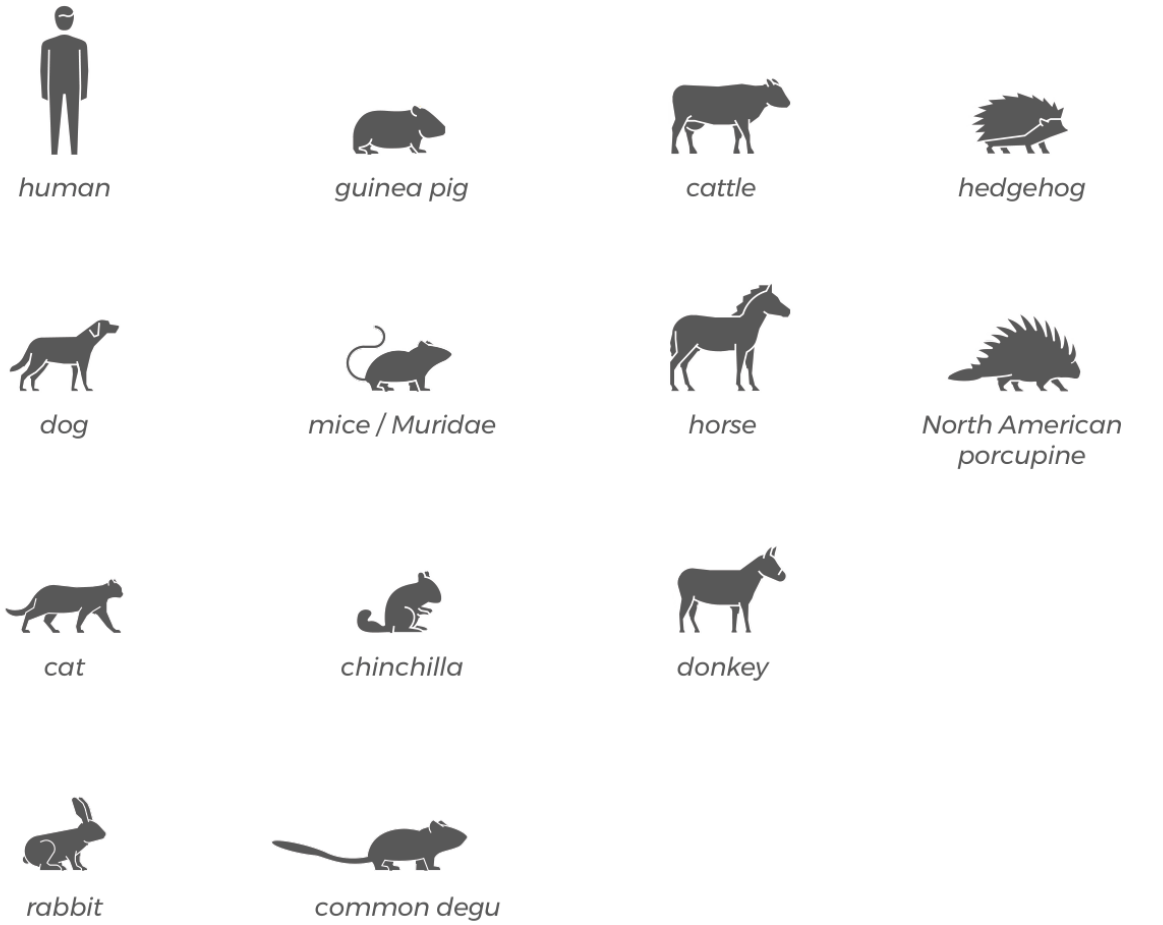
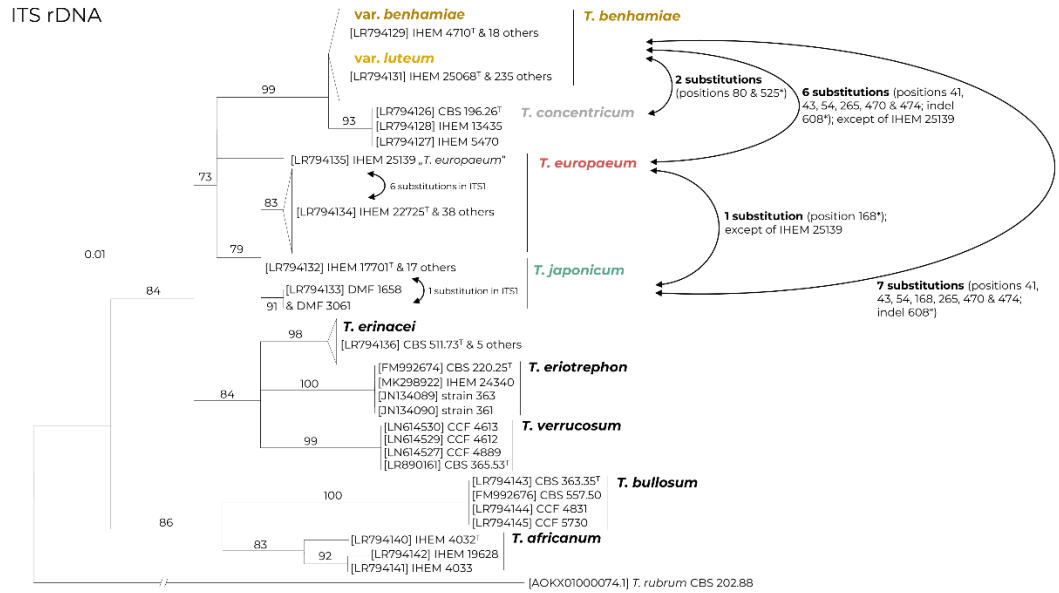
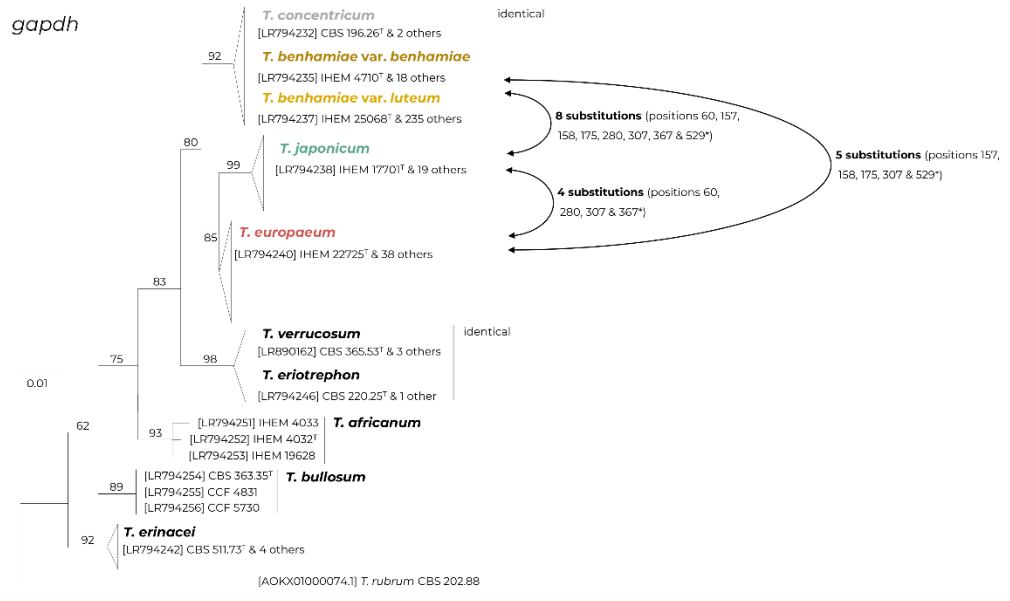


Supplementary file1 **Fig. S1** Legend for the host icons used in this study

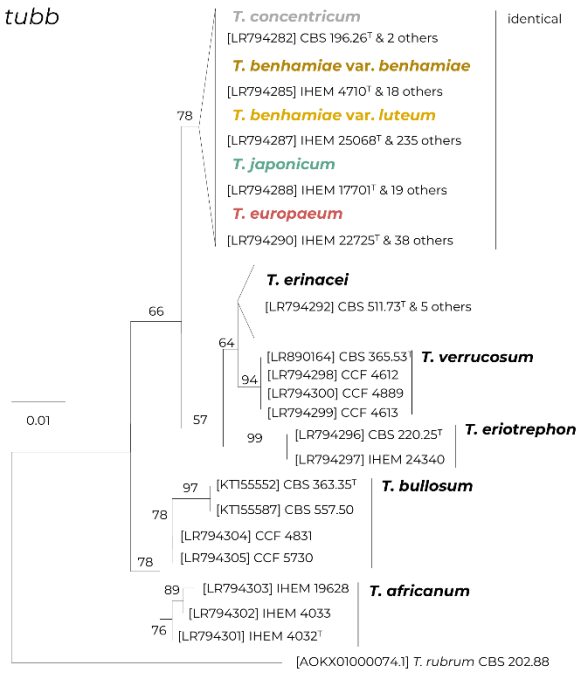


Supplementary file 2 **Fig. S2** Maximum likelihood tree based on ITS region sequences. Maximum likelihood bootstrap values are appended to the nodes; only support values higher than 70% are shown; the ex-type strains are designated with a superscripted T; *Trichophyton rubrum* CBS 202.88 was used as the outgroup. Clades with >5 identical sequences are collapsed; *positions refer to the alignment available in the Supplementary material

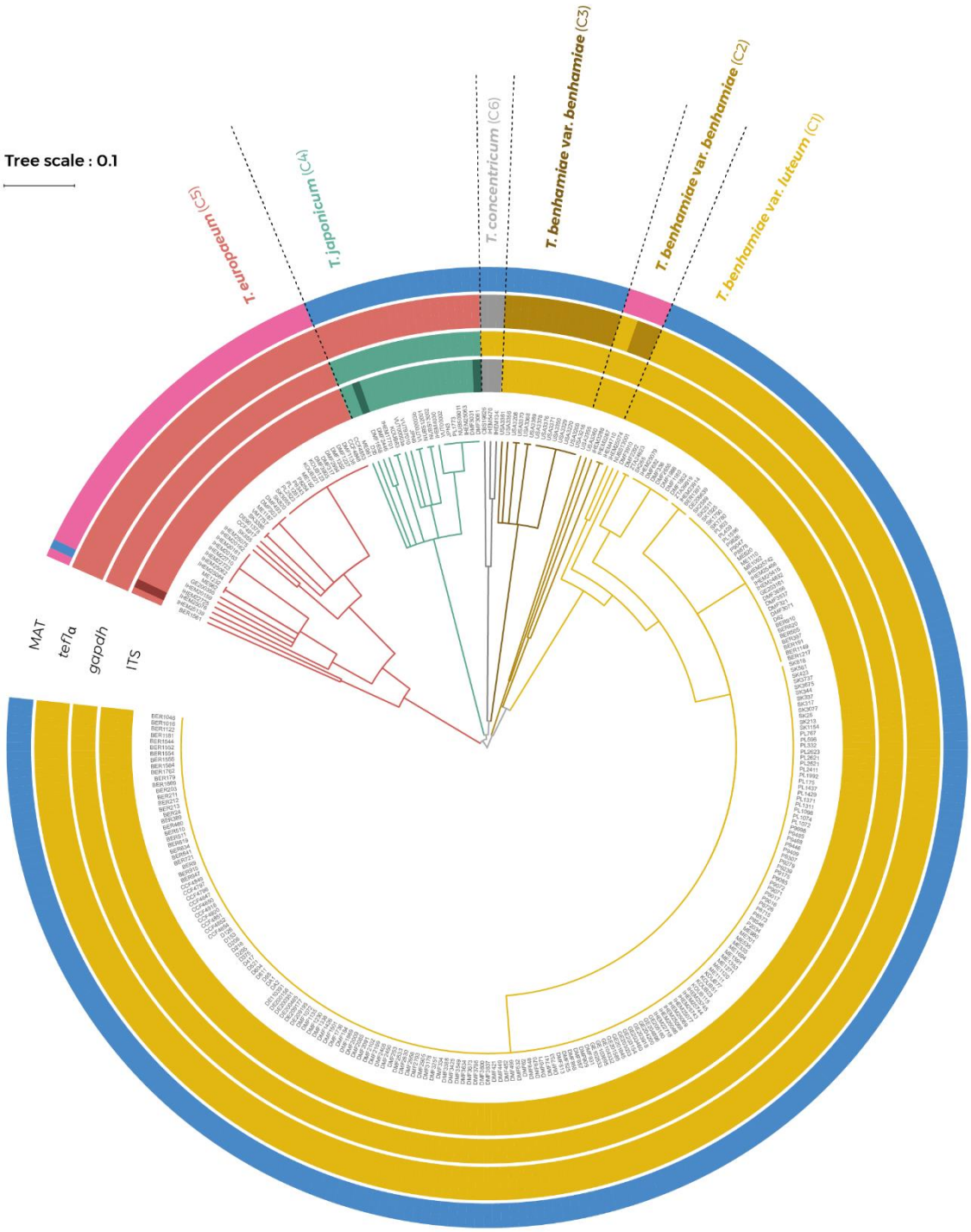


Supplementary file3 **Fig. S3** Maximum likelihood tree based on *gapdh* gene sequences. Maximum likelihood bootstrap values are appended to the nodes; only support values higher than 70% are shown; the ex-type strains are designated with a superscripted T; *Trichophyton rubrum* CBS 202.88 was used as the outgroup. Clades with >5 identical sequences are collapsed; *positions refer to the alignment available in the Supplementary material

Supplementary file 4 **Fig. S4** Maximum likelihood tree based on *tef1-α* gene sequences. Maximum likelihood bootstrap values are appended to the nodes; only support values higher than 70% are shown; the ex-type strains are designated with a superscripted T; *Trichophyton rubrum* CBS 202.88 was used as the outgroup. Clades with >5 identical sequences are collapsed; *positions refer to the alignment available in the Supplementary material



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



Supplementary file 6 **Fig. S6** Phylogenetic tree of the *Trichophyton benhamiae* clade revealed by the analysis of ten microsatellite loci in 318 strains constructed in FAMD software using a Jaccard index-based distance matrix. Coloured circles display the genotype diversity of the ITS, *gapdh* and *tef1-α* loci and the distribution of MAT gene idiomorphs across *Trichophyton benhamiae* clade species

PAPER 2

Describing the remaining species diversity in the complex

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Discovery of new *Trichophyton* members, *T. persicum* and *T. spiraliforme* spp. nov., as a cause of highly inflammatory tinea cases in Iran and Czechia

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ABSTRACT

Pathogens from the *Trichophyton benhamiae* complex are one of the most important causes of animal mycoses with significant zoonotic potential. In light of the recently revised taxonomy of this complex, we retrospectively identified 38 *Trichophyton* isolates that could not be resolved into any of the existing species. These strains were isolated from Iranian and Czech patients during molecular epidemiological surveys on dermatophytosis and were predominantly associated with highly inflammatory tinea corporis cases, suggesting possible zoonotic etiology. Subsequent phylogenetic (4 markers), population genetic (10 markers), and phenotypic analyses supported recognition of two novel species. The first species, *Trichophyton persicum* sp. nov., was identified in 36 cases of human dermatophytosis and one case of feline dermatophytosis, mainly in Southern and Western Iran. The second species, *Trichophyton*

spiraliforme sp. nov., is only known from a single case of tinea corporis in a Czech patient who probably contracted the infection from a dog. Although the zoonotic sources of infections summarized in this study are very likely, little is known about the host spectrum of these pathogens. Awareness of these new pathogens among clinicians should refine our knowledge about their poorly explored geographic distribution.

IMPORTANCE In this study, we describe two novel agents of dermatophytosis and summarize the clinical manifestation of infections. These new pathogens were discovered thanks to long-term molecular epidemiological studies conducted in Czechia and Iran. Zoonotic origins of the human infections are highly probable, but the animal hosts of these pathogens are poorly known. Further research is needed to refine our knowledge about these new dermatophytes.

KEYWORDS Dermatophytosis, Molecular epidemiology, Multigene phylogeny, Skin mycoses, *Trichophyton benhamiae* complex, Zoonotic infections, Zoophilic dermatophytes

INTRODUCTION

Dermatophytes are one of the most common pathogens of animals and humans worldwide (1). Because dermatophytosis is not a life-threatening infection and its health impact can be relatively low, it represents a rather neglected infection in many regions. The infections with mild or moderate symptoms and chronic course are mostly caused by anthropophilic dermatophytes, which are typically localized to body areas that have higher moisture levels or are less accessible for the host immune response, e.g., skin folds, feet, and nails (2–4). On the other hand, zoophilic dermatophytes, when transmitted to a human, usually cause highly inflammatory infections located in sites directly exposed to contact with an infected animal, typically the extremities, face, and trunk (5, 6). These differences are usually attributed to adaptation of pathogens to their primary hosts leading to a balanced immune response, i.e., a mild immune response is usually associated with infections caused by anthropophilic species which coevolved with humans, and the opposite is true for zoophilic species (7–9).

A significant portion of the zoophilic *Trichophyton* species belongs to the *Trichophyton benhamiae* complex, which encompasses nine taxa. These are mostly animal pathogens whose hosts include pets, livestock, and free-living animals (10). The only clearly anthropophilic species in the complex is Trichophyton concentricum, a cause of tinea imbricata (Tokelau) in rural indigenous populations in the tropics (11, 12). In the past, *Trichophyton verrucosum* was the best-known and most studied species from this complex. It is a well-known agent of dermatophytosis in cattle and of zoonotic infections, especially in agricultural workers (13, 14). But with the introduction of new agricultural procedures and vaccination of cattle, it has been almost eradicated in many regions (15, 16). While the number of dermatophytoses due to *T. verrucosum* in Europe decreased significantly in the last decades, the interest of clinicians has been aroused by the emergence of infections due to *T. benhamiae*, whose number has

increased significantly, especially in various European countries but also in Japan (17–20). In humans, *T. benhamiae* is usually the causative agent of tinea corporis and tinea capitis, transmitted mainly from guinea pigs but also from many other minor hosts, such as dogs (mostly reported from North America), rabbits, and various rodents. It was shown that *T. benhamiae* is a complex of several zoophilic species which are partly different in their geographic distribution and host range (10). Apart from *T. benhamiae* sensu lato, the complex includes another emerging pathogen that is associated with hedgehogs, Trichophyton erinacei, which is increasingly reported worldwide due to the growing interest of people in pet hedgehogs (21, 22).

The rise of cultivation techniques at the end of the 19th century represented a boom in describing new dermatophyte species, followed by another, smaller wave due to mating experiments in the 1960s and 1970s (23). Huge molecular studies at the turn of the millennium brought rather a decline in the number of accepted species (24, 25). Since then, only seven novel Trichophyton members have been described, three of which, however, belong to the geophilic genus *Arthroderma* based on the current taxonomy (26–30). Thus, novel primary pathogenic *Trichophyton* species are restricted only to *Trichophyton indotineae* (31), a member of the *Trichophyton mentagrophytes* complex, and three novel zoophilic species belonging to the *T. benhamiae* complex, namely, *Trichophyton europaeum*, *Trichophyton japonicum*, and *Trichophyton africanum* (10).

Thousands of strains were collected during epidemiological studies in Iran and Czechia, and their identification verified based on DNA sequencing. This approach led to the discovery of two new species belonging to the *T. benhamiae* complex that are proposed in this study, using a polyphasic approach comprising morphology, physiology, and molecular data.

RESULTS

Tinea cases due to *Trichophyton persicum*.

In total, 37 cases of dermatophytosis caused by *T. persicum* were identified during retrospective analysis of internal transcribed spacer (ITS), ribosomal DNA (rDNA), and tef1-asequences generated during epidemiological surveys in Iran conducted between 2008 and 2019. One case of feline dermatophytosis was also identified. The cases are summarized in Table 1. Originally, the *T. persicum* isolates were identified by Iranian medical mycologists as *T. verrucosum* or *Microsporum canis*, based on morphology. Anamnestic data about the contact of patients with animals were usually missing. The only exception was the case of a 31-year-old woman (isolate S366) who lived in a rural area of Iran and reported contact with various farm animals, including cats and cattle.

In general, the cases of dermatophytosis due to *T. persicum* were reported especially from the area of Southern, Southwestern, and Eastern Iran (Fig. 1). The first case of dermatophytosis caused by *T. persicum*, from 2011, was traced in Isfahan, followed by 17 cases that originated mainly in Southern

and Southwestern Iran (Shiraz, Yasuj, and Ahvaz) during 2012 to 2014. The last and larger group of cases occurred in the area of Eastern Iran (10 cases in Qaen) during 2017, together with a few cases traced mainly in Shiraz during 2017 to 2019.

Almost all infections due to *T. persicum* manifested as tinea corporis (Table 1), except for a patient with a scalp infection (ectothrix). The exact body area of infections was usually missing in the anamnestic data, but in the cases with known details, the lesions were located mainly on the upper extremities (dorsum of hand, arm, elbow, and wrist) and, less frequently, on the trunk, face, and feet (Fig. 2A to C). The youngest patient was only 2 years old and the oldest 68years old. Even though the patients' age range is wide, young adults (median age 24) were the most frequently affected. Females represented two-thirds of patients, with a median age of 30years, while the median age of male patients was 11years.

Tinea corporis due to *Trichophyton spiraliforme*.

A 43-year-old immunocompetent male patient presented with a painful inflammatory skin change on the inner aspect of his left knee. There was no history of prior trauma to the site. He was otherwise healthy except for a history of congenital hyperbilirubinemia and penicillin allergy. The patient was first treated with roxithromycin 150 mg twice a day orally and ammonium bituminosulfonate ointment (Ichtoxyl ung) topically based on the initial diagnosis of bacterial pyoderma. There was no visible improvement at the 10-day follow-up.

A dermatological examination demonstrated a well-defined 6- by 3.5-cm erythematous pustular plaque with adherent yellowish crusts (Fig. 2D to F). A swab sample was taken from the pustules and crusts for mycological examination. Fungal mycelium was demonstrated on potassium hydroxide examination, and the agent was preliminarily identified based on morphological features as the zoophilic strain of *Trichophyton interdigitale* according to the concept of Heidemann et al. (32). The identification was subsequently corrected to Trichophyton sp. after ITS rDNA region sequencing.

Nobody else in the patient's surroundings had similar symptoms. Oral treatment with terbinafine (250mg daily) in combination with clotrimazole (1% cream, two times a day) was initiated based on the result of the mycological examination. The pustules disappeared in 2weeks after starting the treatment. A cure was achieved 4 weeks after the onset of the treatment, with only slight erythema remaining. No recurrence has been observed to date.

Later the patient mentioned that he owned a dog (German shorthaired pointer) that had been treated with antimycotics at a veterinary surgeon 2 months ago due to a lesion on its front paw above the ankle. Although the transmission of the infection from the dog cannot be confirmed due to the absence of an animal isolate, it is highly suspected.

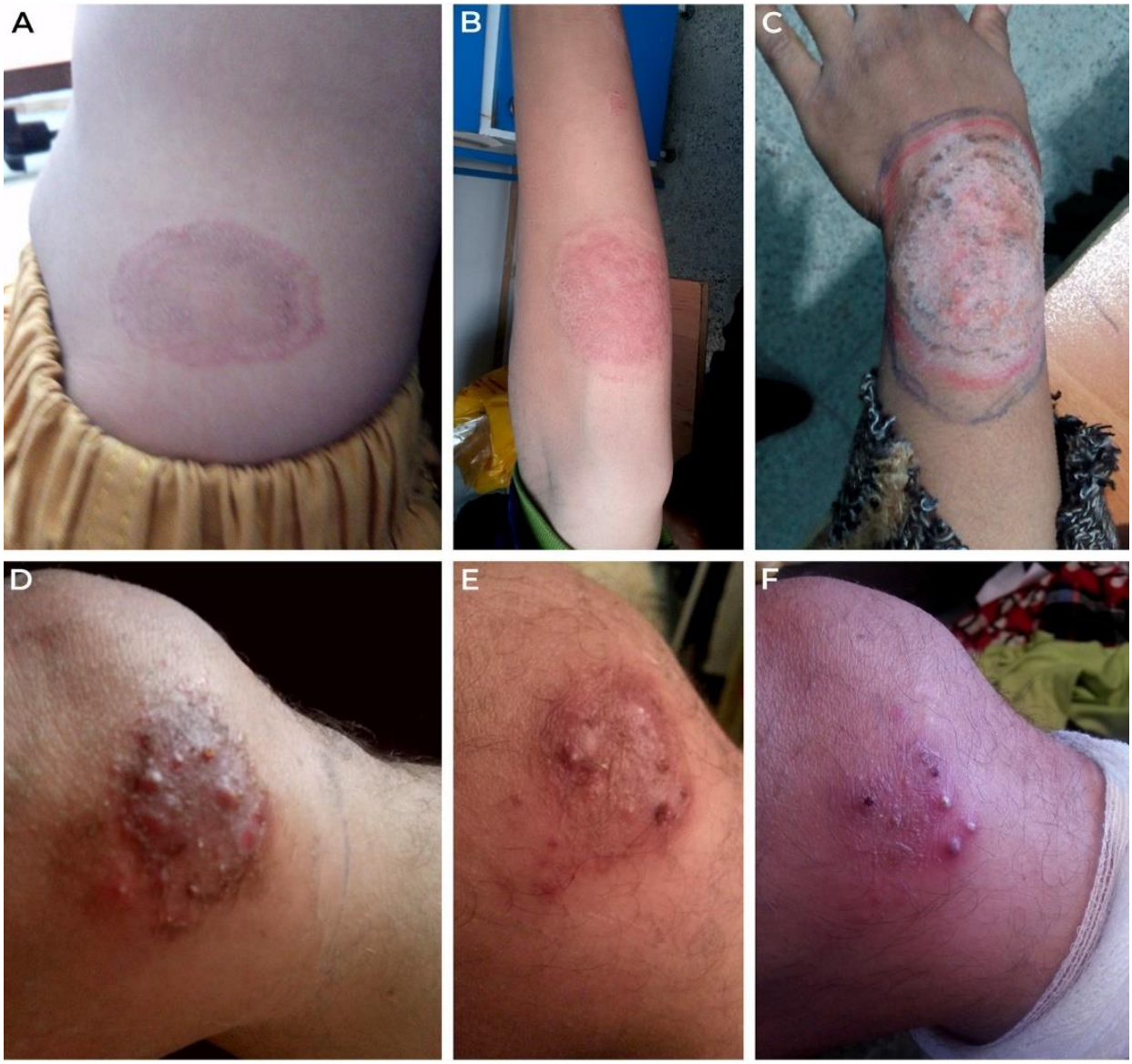

Fig. 2 Clinical manifestations of infections caused by *Trichophyton persicum* (A to C) and *Trichophyton spiraliforme* (D to F). Annular lesion with erythematous raised margins on the abdomen of a 2-year-old boy (isolate Ahv-525) (A); tinea corporis of the extensor surface of the right forearm with inflammatory erythema and scaling in a 16-year-old female (isolate Ahv-523) (B); an extended annular, scaly plaque on the extensoral aspect of the left forearm and wrist in a 31-year-old woman (isolate S366)—the highlighted regions are accentuated borders that had been manipulated by the patient (C); red-brown indurated plaque with several pustules, papules and crusts on the inner aspect of the left knee in a 43-year-old man (D); the same lesion after 1 week of terbinafine treatment (E); detail of pustules in the same patient (F).

Table 1 Overview of tinea cases attributed to the newly described *Trichophyton* species

Species / Strain number	Region / Country	Locality	Sex	Age	Clinical manifestation	Date of isolation	GenBank accession numbers			
							ITS rDNA	*tef1-α*	*gapdh*	*tubb*
Trichophyton persicum										
Ahv-376	South-western Iran	Ahvaz	F	34	tinea corporis	2013	KT192449			
Ahv-200	South-western Iran	Ahvaz	F	68	tinea corporis	2013	KT192448			
Ahv-419	South-western Iran	Ahvaz	F	23	tinea corporis	2013	KT192450			
237	South-western Iran	Ahvaz	F	26	tinea corporis	2012-2014	MW936601			
302	South-western Iran	Ahvaz	F	54	tinea corporis	2012-2014	MW936603			
RM6	Central Iran	Isfahan	F	45	tinea corporis	2011	MW936621			
M6	North-eastern Iran	Mashhad	F	20	tinea corporis	2013	MF850250			
CCF 6543^{T} (mums91B, MNJ-91)	Eastern Iran	Qaen	F	5	tinea capitis (ectothrix)	2017	MW936609	MG356864	MW959142	MW959139
MNJ-104	Eastern Iran	Qaen	M	14	tinea corporis	2017	MW936612			
MNJ-120P (mums120B)	Eastern Iran	Qaen	M	10	tinea corporis	2017	MW936613	MG356866		
MNJ-154 (mums154b)	Eastern Iran	Qaen	M	45	tinea corporis	2017	MW936614	MG356865		
MNJ-155 (mums155B)	Eastern Iran	Qaen	M	12	tinea corporis	2017	MW936615	MG356867		
MNJ-16 (mums16B)	Eastern Iran	Qaen	F	6	tinea corporis	2017	MW936616	MG356860		
MNJ-28 (mums28B)	Eastern Iran	Qaen	F	35	tinea corporis	2017	MW936617	MG356861		
MNJ-32 (mums32B)	Eastern Iran	Qaen	M	9	tinea corporis	2017	MW936618	MG356862		
MNJ-6761	Eastern Iran	Qaen	F	29	tinea corporis	2017	MW936619			
MNJ-81 (mums81B)	Eastern Iran	Qaen	M	5	tinea corporis	2017	MW936620	MG356863		
5	Southern Iran	Shiraz	M	26	tinea corporis	2012-2014	MW936604			
25	Southern Iran	Shiraz	F	31	tinea corporis	2012-2014	MW936602			
58	Southern Iran	Shiraz	F	24	tinea corporis	2012-2014	MW936607			
72	Southern Iran	Shiraz	F	38	tinea corporis	2012-2014	MW936608			
172	Southern Iran	Shiraz	F	34	tinea corporis	2012-2014	MW936599			
174	Southern Iran	Shiraz	F	48	tinea corporis	2012-2014	MW936600			
T286	Southern Iran	Shiraz	F	23	tinea corporis	2017-2019	MW936624			
T289	Southern Iran	Shiraz	F	55	tinea corporis	2017-2019	MW936625			
T290	Southern Iran	Shiraz	F	14	tinea corporis	2017-2019	MW936626			
T321	Southern Iran	Shiraz	F	13	tinea corporis	2017-2019	MW936627			
T322	Southern Iran	Shiraz	M	10	tinea corporis	2017-2019	MN807357			
Ci1947	Northern Iran	Tehran	M	24	tinea corporis	2012	MW936610			
SH1	Northern Iran	Tehran	M	25	tinea corporis	2016	MW936623			
KH-M4	North-western Iran	Meshkin-shahr	—	—	dermatophytosis in a stray cat	2015	MW936611			
Ahv-523	South-western Iran	Yasuj	F	16	tinea corporis	2013	KT192451			
Ahv-525	South-western Iran	Yasuj	M	2	tinea corporis	2013	KT192452			
S366	South-western Iran	Yasuj	F	31	tinea corporis	2013	MN808768			
S394	South-western Iran	Yasuj	F	16	tinea corporis	2013	MW936622			
501	South-western Iran	Yasuj	F	45	tinea corporis	2012-2014	MW936605			
502	South-western Iran	Yasuj	M	10	tinea corporis	2012-2014	MW936606			
Trichophyton spiraliforme										
CCF 6259^{T}	Czech Republic	Hostouň	M	43	tinea corporis	2017	MW936628	MW959141	MW959143	MW959140

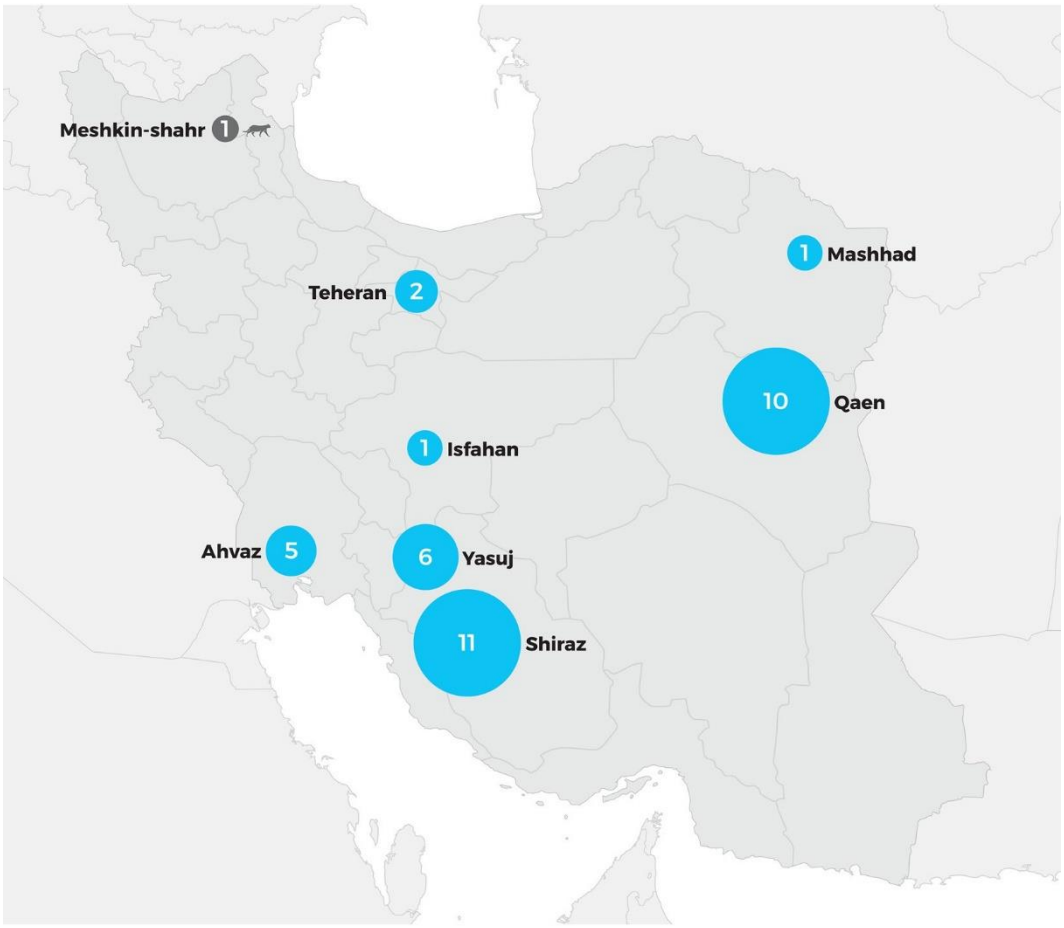

Fig. 1 Geographic map distribution of tinea cases identified as due to *Trichophyton persicum* in Iran.

Phylogeny

For the phylogenetic analysis, we used a previously published alignment consisting of 340 combined ITS, gapdh, tubb, and tef1-asequences from members of the

T. benhamiae complex (10); only the sequences of the presumed hybrid IHEM 25139 were excluded. The alignment was enriched by available ITS and tef1-asequences from *T. persicum*. Four genetic loci were only amplified in the viable ex-type isolates of both *T. persicum* and *T. spiraliforme*. In addition, one Iranian strain of Trichophyton eriotrephon was also added. The final alignment included 378 isolates and 2,374 characters, with 243 variable and 153 parsimony informative sites. The alignments, GenBank accession numbers, and origins of all 378 isolates are available in the Dryad Digital Repository at https://doi.org/10.5061/dryad .59zw3r275.

The phylogenetic analysis resolved both new species within the *T. benhamiae* clade sensu Cmoková et al. (10) of the *T. benhamiae* complex (Fig. 3). *Trichophyton spiraliforme* was placed in the sister position to *T. persicum* with a Bayesian posterior probability of 0.96, while the maximum-likelihood (ML) bootstrap was low (,70%). The relationships of these species with other taxa in the *T. benhamiae* clade are poorly resolved due to insufficient statistical supports, but the closest species are represented by *T.*

japonicum and *T. europaeum*. The phylogenies based on the ITS rDNA region (all isolates) and 4 loci (only isolates having full sequence data available) are shown for comparison in Fig. S1 and S2 in the supplemental material. The topologies of these phylogenetic trees are similar, with some differences in deep nodes that gained low statistical support in all phylogenies.

Trichophyton persicum and *Trichophyton spiraliforme* can be differentiated from each other by their ITS rDNA (2 substitutions in the ITS2 region), gapdh (12 substitutions), and tef1-agene sequences (1 substitution and 1 indel). The ITS rDNA and gapdh loci can also differentiate both species from the other members of the *T. benhamiae* complex. The sequence of the tef1-agene of *T. spiraliforme* is identical to those of *T. europaeum* and *T. japonicum*, and it shows a single substitution and indel compared to that of *T. persicum*. The two new species share identical tubb sequences that differ by two substitutions from those of all other members of the *T. benhamiae* clade. The tubb locus shows overall low discriminatory power in the *T. benhamiae* complex, and all remaining members of the *T. benhamiae* clade have identical tubb genotypes, as demonstrated previously (10).

Some level of intraspecific variability was detected in the available ITS (n = 37) and tef1-a(n = 8) sequences of *T. persicum*; specifically, there were three substitutions and one indel in the ITS region and a single substitution in tef1-a gene sequences. The remaining two loci were only available for the ex-type strains, and consequently, the level of intraspecific variability, along with the precise numbers of species-specific substitutions/indels useful for their differentiation, needs to be confirmed in future studies when more strains will be available. Given the importance of the ITS rDNA region for routine dermatophyte identification, we summarized all species-specific substitutions and indels between all species pairs from the *T. benhamiae* clade in Table S1. The genotype network of the *T. benhamiae* complex based on a combined alignment (only strains with all four loci available) is shown in Fig. 4, where hash marks on the connecting lines indicate the numbers of substitutions between species. Fig. 4 also shows the distribution of MAT1-1-1 and MAT1-2-1 gene idiomorphs across the *T. benhamiae* complex. In general, both idiomorphs are unequally distributed in most species of the complex, and one idiomorph is frequently absent, suggesting clonal spread of the majority of species. Data from more strains are needed for MAT idiomorph distribution assessment in the two newly described species.

Microsatellite analysis.

The microsatellite analysis (Fig. 5) resolved both new species into proximity to *T. benhamiae* var. benhamiae cluster 2 and *T. benhamiae* var. luteum. Both *T. persicum* and *T. spiraliforme* formed independent distant lineages. The analysis separated different subpopulations of *T. benhamiae* var. benhamiae. Cluster 2 of *T. benhamiae* var. benhamiae sensu Cmoková et al. (10) consisted of two lineages related to *T. benhamiae* var. *luteum*, while cluster 1 grouped with *T. concentricum*. Consequently, *T. benhamiae* did not form a monophyletic group. This population genetic variability in *T. benhamiae* is, however, not reflected by standard sequence markers and multigene phylogeny (Fig. 3).

Phenotype analysis

The growth rate of *T. spiraliforme* is similar to the growth rates of other zoophilic species from the *T. benhamiae* clade with the so-called "white phenotype" (*T. europaeum*, *T. japonicum*, and *T. benhamiae* var. *benhamiae*). *Trichophyton spiraliforme* differs mainly by its restricted growth on potato dextrose agar (PDA). Slightly lower growth rates on PDA were also observed in some strains of T. europaeum, but not to such an extent. *Trichophyton persicum* grows restrictedly also but on all media tested, as observed in the ex-type strain. Limited data are available for several strains that were no longer viable and could not be used for detailed analyses. These strains (MNJ-81, S394, and KH-M4) were all characterized by slow growth on Sabouraud dextrose agar (SDA) with chloramphenicol and cycloheximide. Very restricted growth on SDA at 37°C also differentiates *T. persicum* from other closely related zoophilic species. Both new species produce small microconidia whose dimensions do not deviate significantly from those of other *T. benhamiae* clade species (Fig. 6). Microconidia were abundantly produced and predominantly clavate in *T. spiraliforme*, while relatively poor production was observed in *T. persicum* and the conidia were frequently irregular and diverse in shape (Fig. 7). In this aspect, *T. persicum* resembles *T. eriotrephon*. *Trichophyton spiraliforme* produced a large number of spiral hyphae that were observed already in 7-day-old colonies and were very abundant after 21 days of cultivation. Although this is a very subjective character, such an extensive production of spiral hyphae is uncommon in *T. benhamiae* clade species and is rather typical for *T. mentagrophytes*.

Taxonomy

Trichophyton persicum Rezaei-Matehkolaei, Cmokova & Hubka sp. nov. MycoBank accession number MB839323; Fig. 7.

Etymology: referring to the origin of the examined strains, Persia, the ancient name of Iran.

Holotype: PRM 954724, a dried herbarium specimen (isotypes PRM 954725 and PRM 954726); Iran, Qaen, tinea capitis (ectothrix), 5-year-old girl, 2017, M. J. Najafzadeh, ex-holotype living culture CCF 6543=MJN-91.

Vegetative hyphae smooth, septate, hyaline, 1 to 2.5mm in diameter (mean 6 standard deviation [SD], 1.56 0.3). Conidiophores poorly differentiated from vegetative hyphae and represented by conidiogenous hyphae, unbranched or with sparse lateral branches, usually densely septate. Microconidia are rare, borne sessile on hyphae or on short lateral protrusions of hyphae, irregularly shaped, usually clavate, ovoid, or pyriform, less commonly barrel shaped or irregular, occasionally

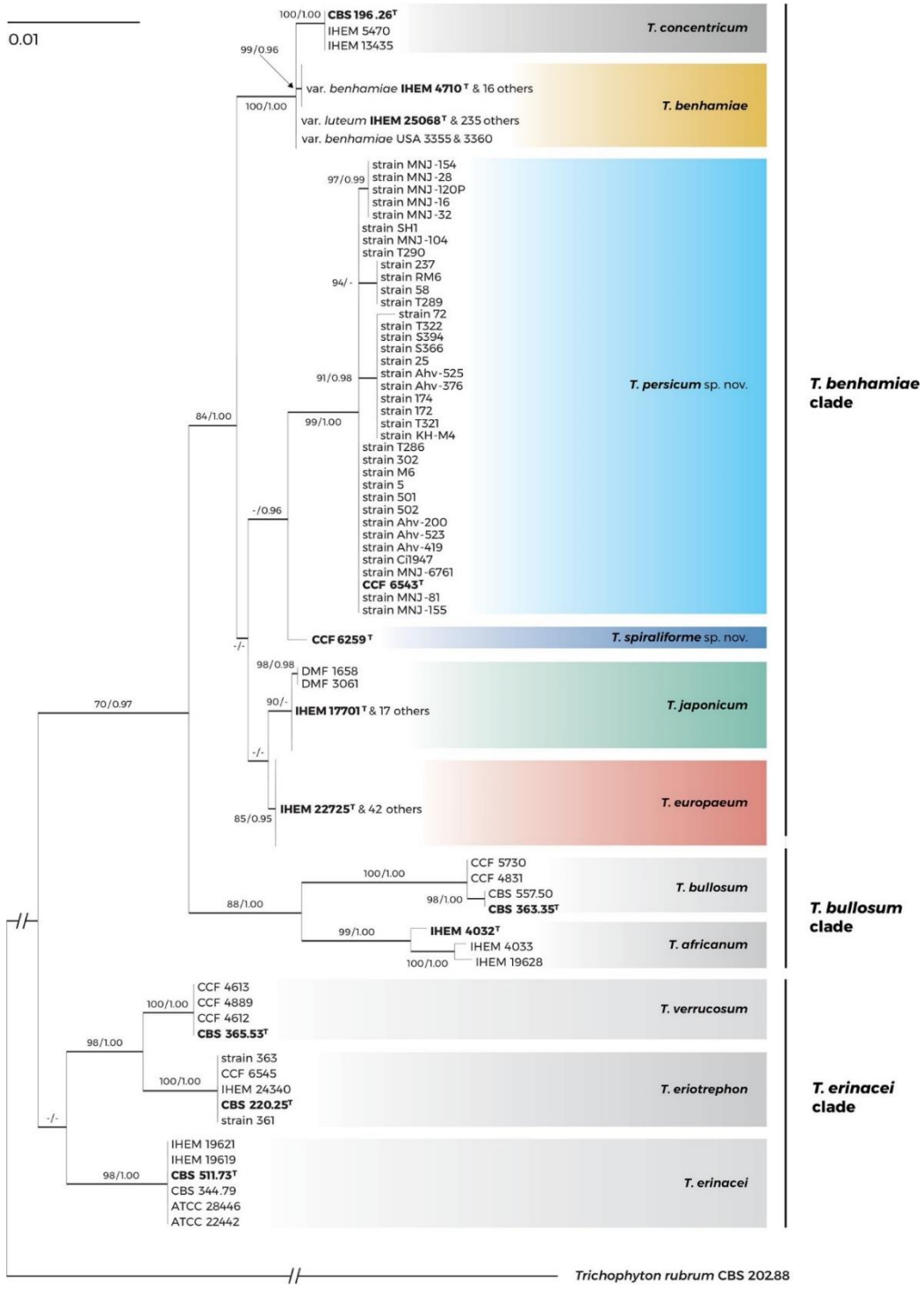

Fig. 3 A best-scoring maximum-likelihood tree (*gapdh*, *tubb*, ITS rDNA, and *tef1-a*) showing relationships of *Trichophyton spiraliforme* sp. nov. and *Trichophyton persicum* sp. nov. to other dermatophytes belonging to the *T. benhamiae* complex. Only support values exceeding bootstrap values of 70% and Bayesian posterior probabilities of 0.90, respectively, are shown. Ex-type isolates are designated by a superscript T. *Trichophyton rubrum* CBS 202.88 was used as the outgroup. The phylogenies based on the ITS rDNA region (all isolates) and 4 loci (only isolates having full sequence data available) are shown in the supplemental material (Fig. S1 and S2) for comparison.

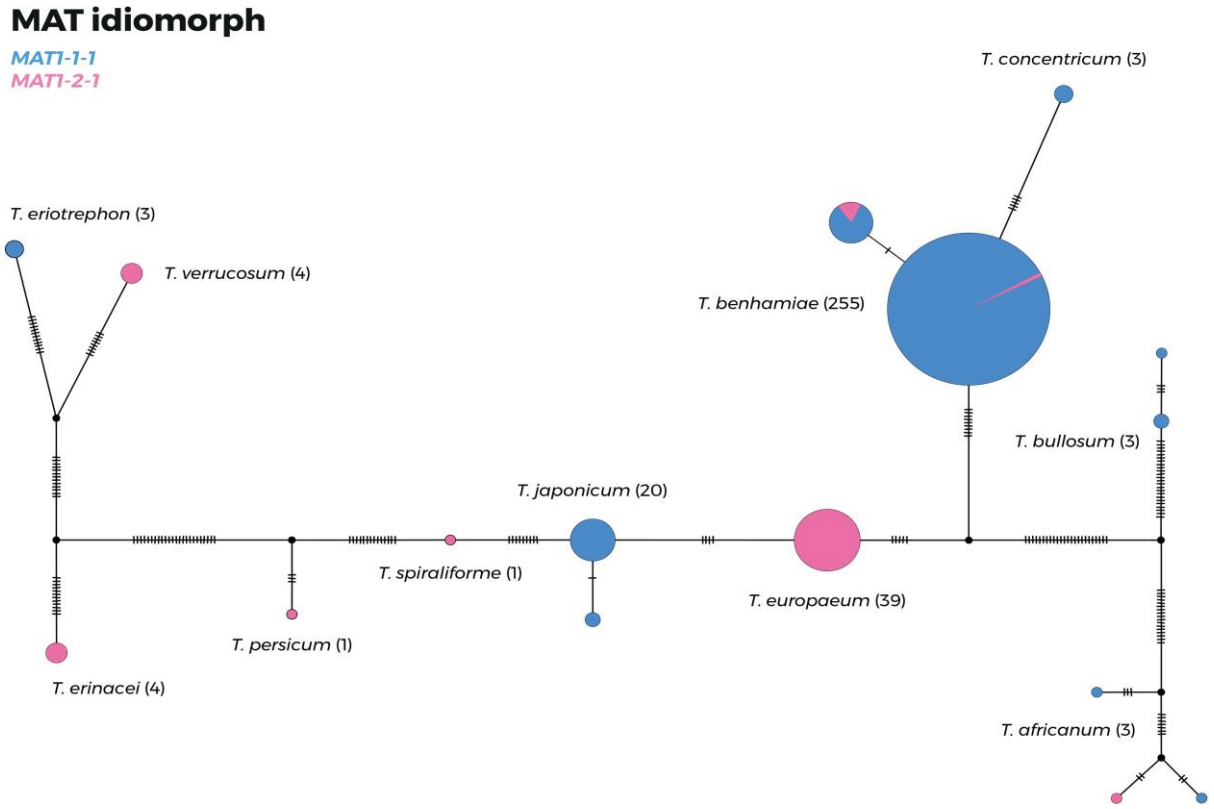

Fig 4 Haplotype network based on multilocus data (ITS rDNA, *gapdh, tef1-a*, and *tubb* loci) showing the distribution of mating-type (MAT) gene idiomorphs in the *Trichophyton benhamiae* complex. Haplotypes are indicated by circles whose sizes correspond to the numbers of strains analyzed (data from Cmoková et al. [10], updated by new isolates from this study), with the numbers of strains given in parentheses after the species name, and hash marks on the connecting lines indicate substitutions (indels are excluded).

released together with fragile lateral protrusions of hyphae, 2 to 6 (3.66 0.7) by 1.5 to 3 (26 0.3)mm. Macroconidia not observed. Chlamydospores present. Spiral hyphae not observed even after 21days of cultivation. Sexual morph is unknown.

Colonies on malt extract agar (MEA; Oxoid, Basingstoke, UK) in 7 days at 25°C attained 9 to 11mm in diameter (ø = 11mm), white (#F5F5F0) to pale orange yellow (#F3E5AB), raised in the center, velvety to cottony, edge entire, reverse light orange yellow (#F8DE7E) to vivid orange yellow (#F6A600). Colonies on SDA in 7 days at 25°C attained 10 to 12mm in diameter (ø = 11mm), white (#F5F5F0), pale orange yellow (#FFF587), or light yellowish brown (#E3D6A1); dark brown diffuse pigment produced into medium after 3 to 4weeks of cultivation. Colonies on PDA in 7 days at 25°C attained 8 to 10mm in diameter (ø = 8mm), white (#F5F5F0) to pale orange yellow (#F3E5AB), raised, velvety or cottony, edge entire, reverse light orange yellow (#F8DE7E). Colonies on SDA in 7 days at 30°C attained 15 to 18mm in diameter (ø = 17mm) and at 37°C attained 5 to 8mm in diameter (ø = 6mm).

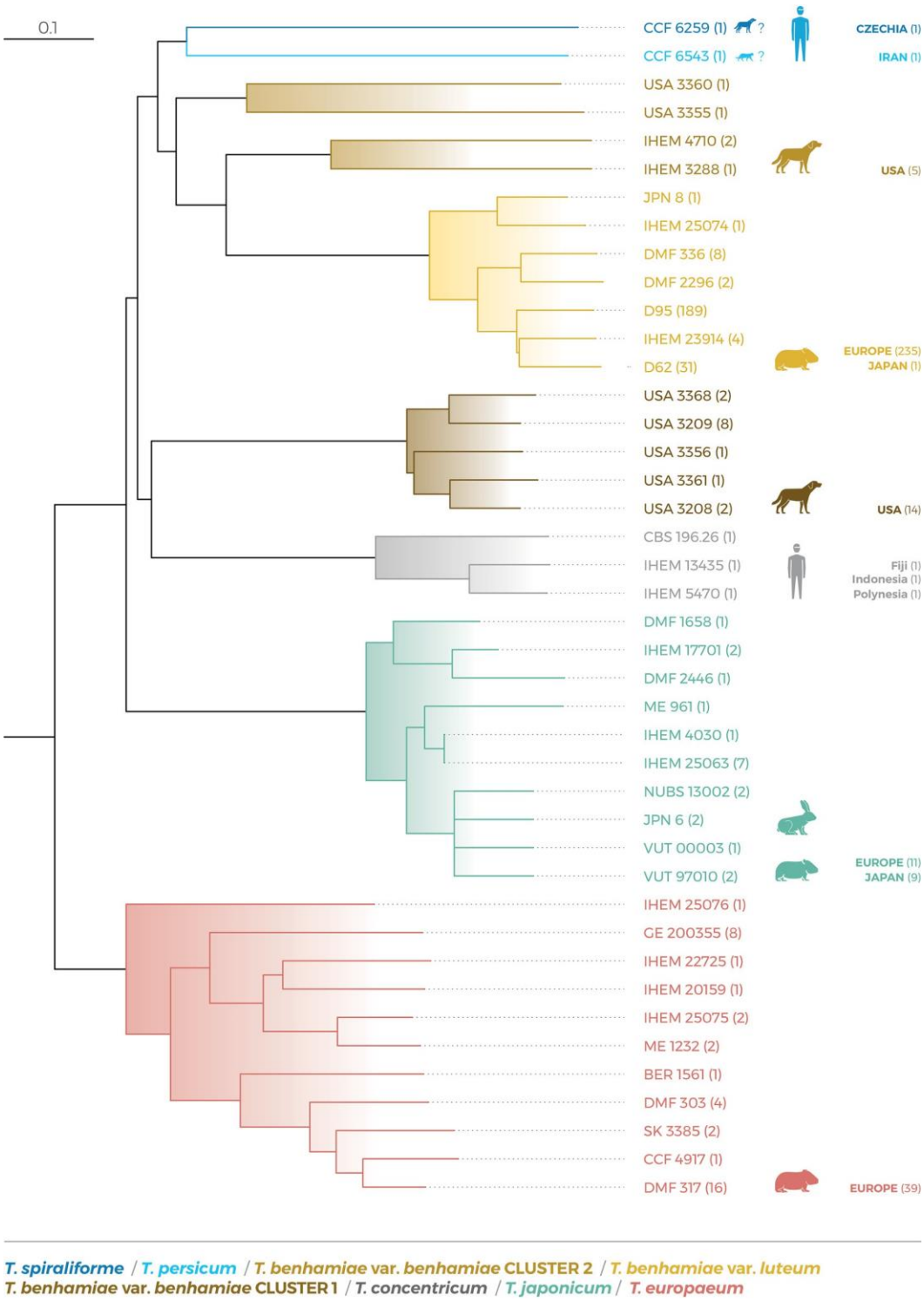

Fig. 5 The population structure of the *Trichophyton benhamiae* clade, based on 10 microsatellite loci and 319 isolates (data from Cmoková et al. [10], updated by new isolates from this study). The neighbor-joining tree was calculated from the multilocus microsatellite profiles using the Jaccard distance matrix measure in FAMD 1.3 (86). The assignment of strains to main clusters and species is indicated by different colors; clones were discarded from the analysis. The number of isolates representing each haplotype is indicated in parentheses following the isolate number. The labels of each cluster show the geographic origin of strains with the total number of isolates and the main primary host(s).

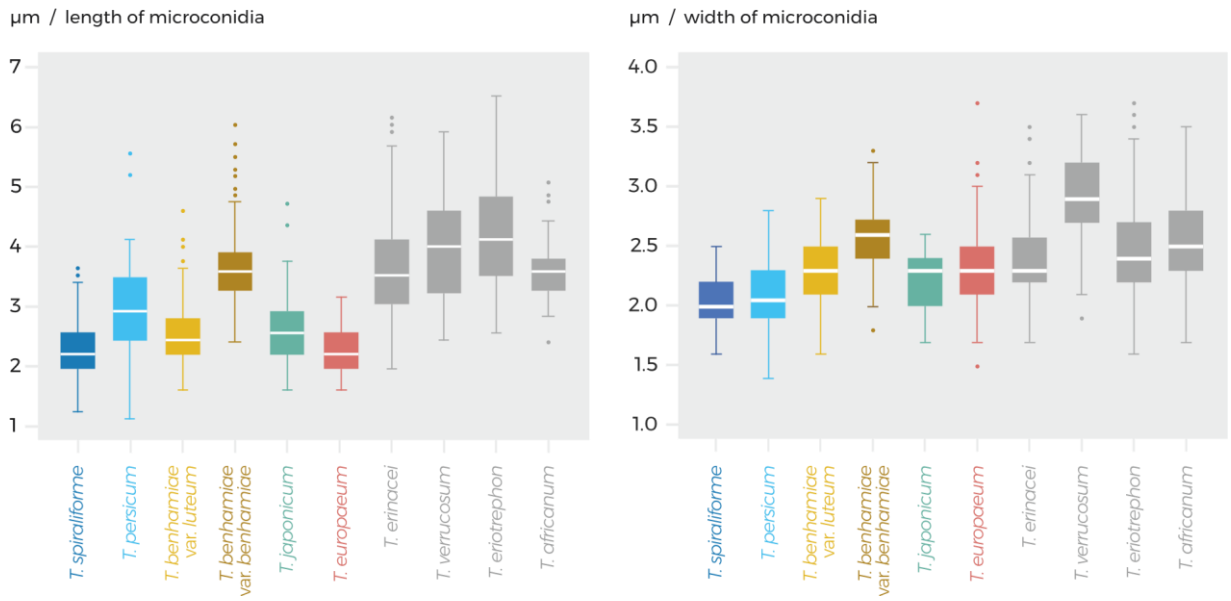

FIG 6 Lengths and widths of microconidia in taxa belonging to the Trichophyton benhamiae complex, including two new species, T. spiraliforme and T. persicum. The horizontal lines indicate mean values and interquartile ranges, whiskers span the 5% and 95% percentiles, and circles represent extreme outliers.

Trichophyton persicum differs from zoophilic members of the *T. benhamiae* clade by its low growth rates on all media examined and restricted growth at 37°C. Other slow-growing species in this clade are the anthropophilic *T. concentricum* and zoophilic *T. benhamiae* var. luteum. The latter differs from *T. persicum* by a yellow colony reverse, the shape of the microconidia, and ecology. *Trichophyton persicum* most closely resembles *T. eriotrephon* by the variability in the shape of microconidia and restricted growth on 37°C. These species can be differentiated by conidium length, which is longer in *T. persicum* than in *T. eriotrephon*. The macromorphology of *T. persicum* may resemble that of *T. verrucosum* by slow growth and colony appearance, but the species differ in micromorphology, as *T. verrucosum* usually produces only chlamydospores and no microconidia. The only viable isolate examined by us exhibited a MAT1-2-1 mating type gene idiomorph.

Trichophyton spiraliforme Cmokova, Kuklova & Hubka, sp. nov. MycoBank accession number MB839324; Fig. 8.

Etymology: referring to the abundant production of spiral hyphae.

Holotype: PRM 954616, a dried herbarium specimen (isotype PRM 954617); Czech Republic, Hostouň near Prague, knee skin (tinea corporis profunda), 43-year-old man, 2017, J. Stará & I. Kuklová, ex-holotype living culture CCF 6259 = SK 4179/17.

Vegetative hyphae smooth, septate, hyaline, 1 to 3.5mm in diameter (mean 6 SD, 2.2 6 0.7). Conidiophores both poorly differentiated from vegetative hyphae and represented by conidiogenous hyphae with sparse to numerous short lateral branches, occasionally well differentiated and branched in a pyramidal (grape-like) pattern. Microconidia abundant, pyriform to clavate, 2.5 to 4 (3 6 0.4) 2 to 2.5 (2 6 0.2) mm. Macroconidia rare, borne laterally or terminally on hyphae, consisting of 5 to 8 cells (median = 5), 25.5 to 33.5 (26.8 6 3.5) by 4.3 to 6 (5 6 0.6) mm, elongated, cigar-shaped, clavate, with a tapering rounded apex and truncate base. Chlamydospores present. Spiral hyphae observed after 7 days of cultivation, abundantly present after 21 days. Sexual morph is unknown.

Colonies on MEA in 7 days at 25°C attained 15 to 35 mm in diameter (ø = 21 mm), granular, white (#F2F3F4) to light yellow (#FAD6A5), flat, edge irregular, submerged, reverse pale orange yellow (#FAD6A5) to brilliant orange yellow (#FFC14F). Colonies on SDA in 7 days at 25°C attained 14 to 17mm in diameter (ø = 15mm), white (#F2F3F4), powdery, umbonate, edge irregular, submerged, reverse deep brown (#593319) in the center, light orange yellow (#FBC97F) in the marginal parts. Colonies on PDA in 7 days at 25°C attained 8mm in diameter (ø = 8mm), white (#F2F3F4) to light yellow (#FAD6A5), granular, occasionally with filamentous sectors, raised, reverse pale orange yellow (#FAD6A5) to brilliant orange yellow (#FFC14F). Colonies on SDA in 7 days at 30°C attained 25 to 27mm in diameter (ø = 26mm) and at 37°C attained 20 to 24mm in diameter (ø = 22mm).

The morphology of *T. spiraliforme* most closely resembles those of related species from the *T. benhamiae* complex (*T. europaeum*, *T. japonicum*, and *T. benhamiae* var. *benhamiae*) and *T. mentagrophytes* by colony texture and numerous relatively small microconidia borne on conidiophores branched in a grape-like pattern. It differs from the species mentioned by its restricted growth on PDA. The only isolate examined by us exhibited a MAT1-2-1 mating type gene idiomorph.

DISCUSSION

In our previous research, we used a polyphasic approach based on morphology, physiology, ecology, clinical picture, and molecular data, including noncoding regions, to resolve the taxonomy of the *T. benhamiae* complex (10, 33). This approach supported the inclusion of nine taxa in the complex, including three new species and one new variety. A similar approach was applied to resolve the positions of tentative new species in this study and led to the description of *T. persicum* and *T. spiraliforme*. While the DNA sequence data assigned both species to the vicinity of *T. europaeum* and *T. japonicum* (Fig. 3), microsatellite data rather showed their relatedness with North American strains of *T. benhamiae* var. benhamiae cluster 2 (Fig. 5), mostly isolated from dogs. Based on microsatellite data, North American strains of *T. benhamiae* var. *benhamiae* do not form a monophyletic group due to high variability, which

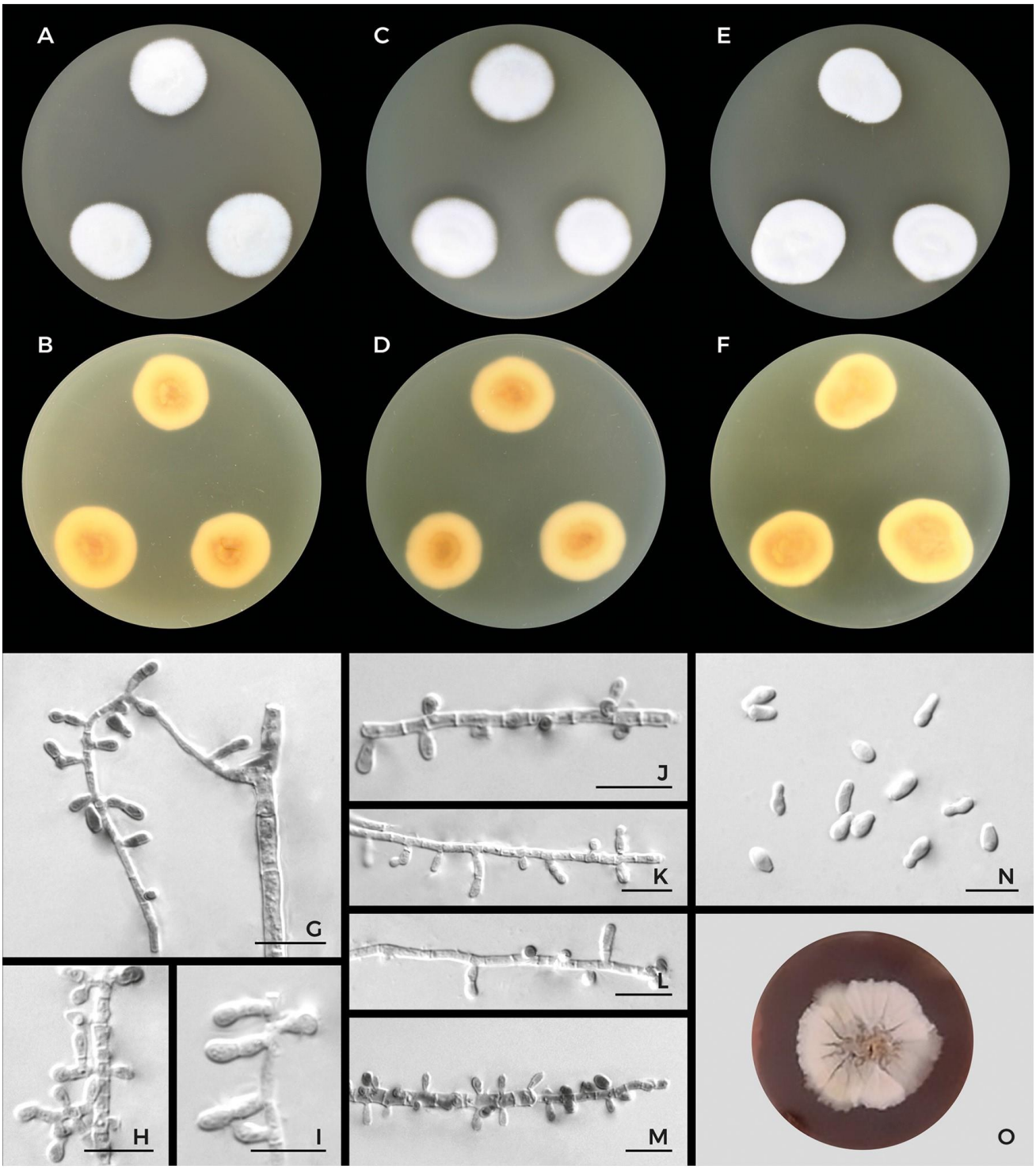

Fig. 7 Macromorphology and micromorphology of *Trichophyton persicum* CCF 6543. Colonies after 2 weeks of cultivation at 25°C on SDA (A) and the reverse (B), on MEA (C) and the reverse (D), and on PDA (E) and the reverse (F); conidiophores (G to M); microconidia and intercalary conidia with varying shapes (N); and a colony with brown diffuse pigment produced into the medium (strain MJN-16) after 2 weeks at 25°C on SDA supplemented with cycloheximide and chloramphenicol (O). Scale bars, 5mm.

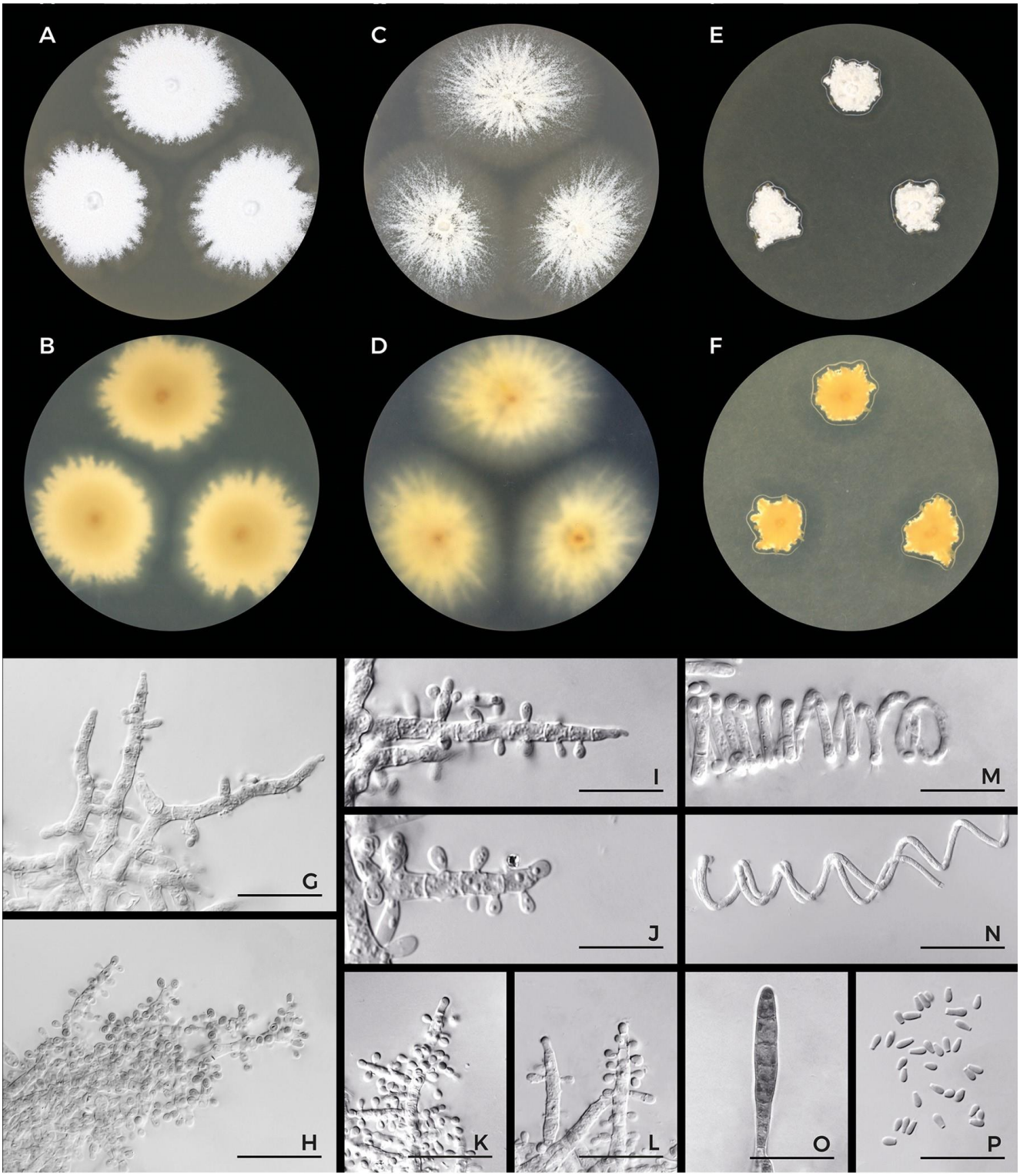

Fig. 8 Macromorphology and micromorphology of *Trichophyton spiraliforme* CCF 6259. Colonies after 2 weeks of cultivation at 25°C on SDA (A) and the reverse (B), on MEA (C) and the reverse (D), and on PDA (E) and the reverse (F); conidiophores (G to L); spiral hyphae (M and N); macroconidium (O); and microconidia (P). Scale bars, 10mm.

is not reflected in standard DNA sequence markers. Thorough sampling and obtaining a larger number distant and independent lineages separated from each other and the population of *T. benhamiae* of North American *T. benhamiae* strains, as well as *T. spiraliforme* and *T. persicum* strains, would probably better elucidate the population structure of the whole species complex. Because only one strain each of *T. spiraliforme* and *T. persicum* was available for microsatellite analysis, we cannot draw any conclusions about the population structure of these species. However, they formed distant and independent lineages separated from each other and the population of *T. benhamiae*.

Reliable species identification of these new species can be achieved by sequencing of ITS rDNA (Table S1) and gapdh loci, as they contain species-specific substitutions. Identification can also be complemented by routine morphological examination, because the new species show unique phenotypic features. Namely, both species have specific growth parameters: *T. spiraliforme* grows slowly on PDA, while *T. persicum* grows slowly on all media examined at 25°C and grows poorly at 37°C. In addition, *T. persicum* has the characteristic shape of microconidia and sporulates poorly, which is rather atypical for zoonotic pathogens in *T. benhamiae* complex. Other features, such as extensive and early production of spiral hyphae in *T. spiraliforme* or production of pigments into medium, are rather subjective and should be verified on a larger number of strains in the future. Selected characteristics useful for differentiation of particular species in the *T. benhamiae* complex are shown schematically in Fig. 9.
The rise of antifungal resistance in some dermatophytes, especially terbinafine resistance in members of the T. mentagrophytes and Trichophyton rubrum complexes, is a significant clinical problem nowadays, since it can lead to treatment failure (34–36). No antifungal resistance has been reported in members of the *T. benhamiae* complex (37). Several *T. persicum* strains have been tested in previous studies under the name *T. benhamiae* (38, 39) and reidentified in this study. The results showed that the antifungal susceptibility pattern of *T. persicum* does not differ from those of other species of the *T. benhamiae* clade. Luliconazole, efinaconazole, and terbinafine were the most potent antifungals against species from the *T. benhamiae* complex (37). Successful treatment of tinea corporis due to *T. spiraliforme* by terbinafine in this study also indicates susceptibility to this commonly used antifungal agent.

The epidemiology of dermatophytosis in Iran shows local specifics in comparison with its epidemiology in European or Asian countries (40). Compared to European countries, where onychomycosis and tinea pedis are the most common clinical entities and older age groups usually predominate among patients (1), typical Iranian patients belong to younger age groups, and tinea corporis (including tinea cruris) and then scalp infections are the most prevalent types (39, 41, 42). Onychomycosis and tinea pedis contribute to lesser extents to the overall spectrum of dermatophytosis in Iran, but they start to prevail in the Northern, Central, and Western regions (43–47). Aside from rare cases due to *T. verrucosum* and *T. eriotrephon*, zoophilic species from the *T. benhamiae* clade (*T. benhamiae*, *T. europaeum*, *T. japonicum*,

and *T. persicum*) are marginal infectious agents that contribute to the spectrum of all dermatomycosis agents only at rates of 0.5 to 5%. Based on long-term epidemiological data and the sequence data available in the GenBank database, *T. persicum* seems to be the dominant species from the clade in Iran, responsible for almost all cases and mostly restricted to a relatively small area in South, Southwestern, and Eastern Iran (Fig. 1). But it must be added that no extensive epidemiological studies have been performed in neighboring countries. In terms of case frequencies, *T. persicum* is followed by *T. eriotrephon* and then by extremely rare cases due to *T. benhamiae*, *T. europaeum*, and *T. japonicum* (39, 42, 48, 49) which were retrospectively identified based on the data deposited in GenBank (10).

The epidemiological situation in Europe in terms of the occurrence of T. benhamiae complex pathogens is in striking contrast to that in Iran. *Trichophyton benhamiae* var. *luteum* belongs to the most important zoonotic agents of dermatophytosis (19, 20, 50, 51), and cases caused by other species, such as *T. europaeum* and *T. japonicum*, are also relatively common (10). These differences are probably caused by local socioeconomic and cultural practices and different spectra of farmed and pet animals that influence the prevalences of particular species. Although many dermatophytes have global distribution, there are some species restricted to specific geographical areas or with poorly known distribution. Such a phenomenon is well documented, for instance, in species from the *T. rubrum* complex, *Microsporum ferrugineum*, and *Microsporum audouinii* (40, 52–54). There are also several examples in the *T. benhamiae* complex; namely, *T. benhamiae* var. *luteum*, *T. europaeum*, and *T. japonicum* have their main distribution areas in Eurasia, whereas *T. benhamiae* var. *benhamiae* seems to be mostly restricted to North America and *T. concentricum* is typical for tropical regions of Oceania, Southeast Asia, and Central and South America (10). However, international travel and animal transport erase to a large extent the original geographic areas of distribution. *Trichophyton spiraliforme* is apparently a rare species in the human clinical material, at least in Europe, where we have large amounts of molecular data available from multiple surveys (17, 19, 50, 55). On the other hand, it is not known how frequent it can be in veterinary samples where we only have limited data.

It is assumed that both new species are zoophilic pathogens based on their phylogenetic relationships with other zoophilic species and clinical manifestations in human patients. Both pathogens were predominantly isolated from cases of human tinea corporis. In the case of *T. spiraliforme*, a dog is suspected to be the source of dermatophytosis in the patient examined, while in *T. persicum*, there is evidence in one case of isolation from a stray cat.

In general, the spectrum of dermatophytes in dogs is poorly known in Czechia. Data available from other European countries show that canine dermatophytosis is mainly caused by *Microsporum canis*, *T. mentagrophytes*, or Nannizzia spp. (56–60). In general, cases of canine dermatophytosis by *T. benhamiae* complex are rarely reported in Europe, and the majority of cases were described in North America and caused by *T. benhamiae* var. *benhamiae* (10, 61). This variety is related to *T. spiraliforme* based on microsatellite data, which further supports the hypothesis about transmission from a dog.

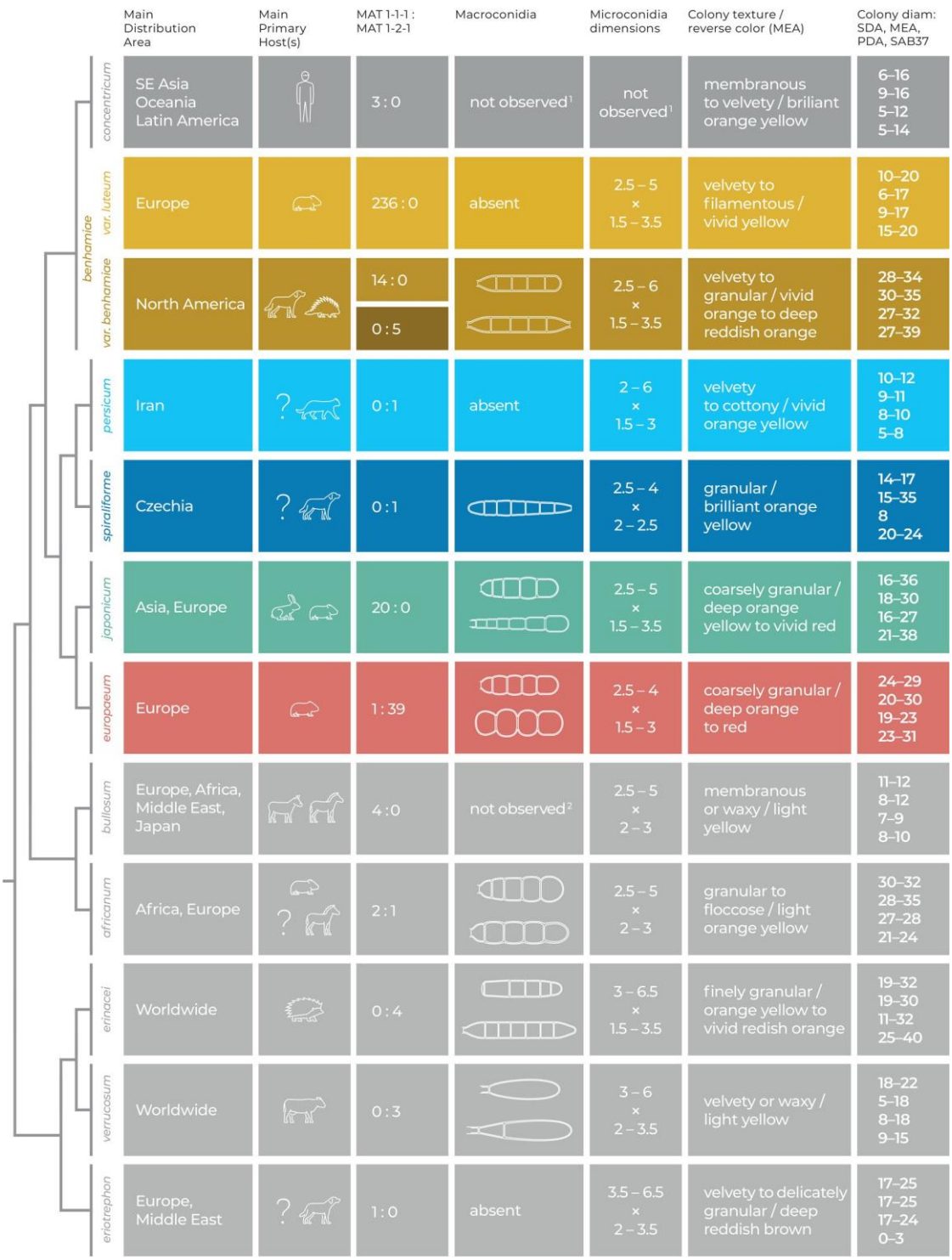

Fig. 9 Overview of selected phenotypic features and ecological data plotted on the simplified phylogeny of the *Trichophyton benhamiae* species complex. The main primary host(s) of particular species are shown as icons; uncommon occasional hosts are omitted; a question mark means that the host spectrum is little known and is based on only one or few isolations from animals/humans. Explanation of superscript numbers: 1, macroconidia were observed in more than one study (12, 88); 2, macroconidia were observed by Lebasque (89) under specific conditions.

Microsporum canis is the main cause of feline dermatophytosis worldwide, and some areas of Iran are not an exception. This pathogen is responsible for up to 95% of feline infections in some studies, and has a prevalence of 26% in asymptomatic cats in Iran. The remaining cases are usually attributed to *T. mentagrophytes*, *T. verrucosum*, and *N. gypsea* (62–66). However, *T. verrucosum* has been reported in other studies as the main cause, with prevalences of up to 14.5% in asymptomatic cats (67). Feline dermatophytosis due to *T. verrucosum* is very unusual, and there are just a few cases reported from other countries besides Iran (68). Although it is known that this species, typically associated with cattle and other ruminants, can sporadically infect any mammal and even birds (22), it could be expected that the course of infections should be rather serious than asymptomatic in cats. There could be several explanations. The isolation from an asymptomatic cat may indicate fomite carriage from exposure to a contaminated environment. Alternatively, we may assume that some cases of feline dermatophytosis could be caused by the slow growing and superficially somewhat similar *T. persicum* and not by *T. verrucosum*, because almost all feline isolates in previous studies from Iran were solely identified by morphological characteristics. The only *T. persicum* isolate from cats examined by us originates from the study of Moosavi et al. (67), who recovered 15 dermatophyte isolates from the skin and hair of 103 asymptomatic stray cats in Meshkin-shahr (Northwestern Iran). Among these strains, 13 were designated as *T. verrucosum* and 2 as T. mentagrophytes based on macro- and micromorphological characters. One *T. verrucosum* isolate was subsequently subjected to ITS rDNA sequencing and has now been found to be identical with *T. persicum* and not *T. verrucosum*.

Conclusion. The increasing number of epidemiological studies substantiated by species identification using molecular data and the fast-growing amount of sequence data in the global databases bring valuable information about ecology, host spectra, and distribution of dermatophyte species that can also lay the groundwork for taxonomic studies. In this study, we were able to retrospectively identify and describe two new *Trichophyton* species based on long-term epidemiological studies on dermatophytosis that were carried out in Iran and Czechia. These presumably zoophilic members of the *T. benhamiae* complex were proposed based on a polyphasic approach, and diagnostic options for their identification are presented based on both molecular and morphological characters. Further research is needed to refine our knowledge about the host spectrum and geographical distribution of these species.

MATERIALS AND METHODS

Strain studies.

We conducted retrospective identification of Czech and Iranian isolates belonging to the *T. benhamiae* complex according to the recent taxonomy (10). All strains were isolated during epidemiological surveys investigating the spectrum of dermatophytosis in different areas in Iran and Czechia during the years 2008 to 2020. Dermatophytosis agents were isolated from skin, nails, nail debris, and scalp samples and identified using molecular methods. In general, ITS rDNA barcode sequences were used for identification, and in addition, a partial tef1-agene, encoding translation elongation factor 1-a, was

amplified in some Iranian strains. Detailed methodologies were described in the particular studies from which the isolates originated: epidemiological surveys were conducted in Tehran during 2008 to 2010 and 2012 to 2013 (44, 45), Shiraz during 2017 to 2019 (39), Southern Iran during 2012 to 2014 (42), Mashhad (North-Eastern Iran) during 2014 to 2015 (48), Isfahan during 2011 to 2012 (69), Khuzestan province during 2013 to 2014 (49), Qaen in 2017 (unpublished), and the various regions in Czechia during 2011 to 2020 (19, 70).

Ex-type isolates of the new species were deposited into the Culture Collection of Fungi (CCF), Department of Botany, Charles University, Prague, Czech Republic, and dried herbarium specimens (holotypes and isotypes) were deposited into the herbarium of the Mycological Department, National Museum in Prague, Czech Republic (PRM).

Molecular studies.

In this study, the molecular studies were restricted to viable isolates, which were represented by ex-type strains of the newly described species. The genomic DNA was extracted from 7day-old colonies using a fungal/bacterial miniprep kit (Zymo Research, Irvine, CA, USA). The quality was evaluated by using a NanoDrop 1000 spectrophotometer. The ITS rDNA region (ITS1–5.8S–ITS2 cluster) was amplified using the primer set ITS1F and ITS4 (71, 72), a partial gapdh gene, encoding glyceraldehyde-3-phosphate dehydrogenase, was amplified with primers GPDF and GPDR (73), a partial tubb gene, encoding b-tubulin, with primers Bt2a and Bt2b (74), and a partial tef1-agene with primers EF-DermF and EF-DermR (75). The primer pair MF3 and MF6 was used for the detection of the MAT1-1-1 idiomorph of the mating type gene (MAT), encoding a protein with an alpha domain motif, and primers TmHMG3S and TmHMG3R were used for the detection of the MAT1-2-1 idiomorph, encoding a regulatory protein with an HMG (high-mobility group) DNA-binding motif (76, 77). The reaction mixture volume of 20 ml contained 1 ml (50 ng ml^{21}) of DNA, 0.3 ml of both primers (25 pM ml^{21}), 0.2 ml of MyTaq polymerase, and 4ml of 5 MyTaq PCR buffer (Bioline, London, UK). The PCR conditions followed the protocol described by Hubka et al. (78). PCR product purification followed the protocol of Réblová et al. (79). Automated sequencing was performed at Macrogen Sequencing Service (Amsterdam, The Netherlands) using both terminal primers.

Phylogeny.

Alignments of the ITS, gapdh, tubb, and tef1-a regions were performed using the FFTNS-i option implemented in MAFFT online (80). The alignments were trimmed and concatenated and then analyzed using maximum-likelihood (ML) and Bayesian inference (BI) methods. Suitable partitioning schemes and substitution models (Bayesian information criterion) were selected using a greedy strategy implemented in PartitionFinder 2 (81), with settings allowing introns, exons, codon positions, and segments of the ITS region to be independent data sets. The partitioning scheme (4 partitions) and substitution models for the ML analysis were as follows: the TrNef1G model was proposed for ITS1

and ITS2; the JC model for the 5.8S region, 1st-codon positions of tubb, and 2nd-codon positions of gapdh, tubb, and tef1-a; the F811I model for the 1st-codon positions of gapdh and tef1-a; and the HKY1I model for the 3rd-codon positions of gapdh, tef1-a, and tubb. The ML trees were constructed with IQTREE version 1.4.4 (82), with nodal support determined by nonparametric bootstrapping with 1,000 replicates. Bayesian posterior probabilities were calculated using MrBayes 3.2.6 (83). The optimal partitioning scheme (4 partitions) and substitution models were identical to the ML analysis, with the exception of the first partition, for which the K801G model was selected. The analysis ran for 10^7 generations, two parallel runs with four chains each were used, every 1,000th tree was retained, and the first 25% of trees were discarded as burn-in. The convergence of the runs and effective sample sizes was checked in Tracer version 1.6. The trees were rooted with *Trichophyton rubrum*.

Haplotype network. A haplotype network was constructed based on the combined ITS, gapdh, tubb, and tef1-a alignment; only isolates with full data available were retained. Variable position were extracted from the combined alignment and the TCS algorithm (84) implemented in the program PopART (85) was used to generate the haplotype network.

Microsatellite analysis.

A multiplex panel consisting of 10 microsatellite markers developed for members of the *T. benhamiae* clade was used (10). The PCR mixture volume of 5 ml contained 50ng of genomic DNA, 0.5ml of a mixture of primers (final concentration 0.1 mM each primer), and 2.5ml of multiplex PCR master mix (Qiagen, Hilden, Germany). The PCR conditions followed the manufacturer's instructions. The PCR products (diluted in water in a 1:20 ratio) were mixed with 10 ml of deionized formamide and 0.2 ml of the GeneScan 600 LIZ size standard and denatured for 5 min at 95°C, followed by analysis on an ABI 3100 Avant genetic analyzer. A binary and allele data matrix was created using GeneMarker 1.51 software (SoftGenetics, LLC, State College, PA, USA) and used to estimate the similarities between individuals using Jaccard's similarity coefficient calculation in the program FAMD (86). A neighbor-joining tree based on Jaccard's similarity coefficient matrix was constructed using the same software.

Phenotype.

Micromorphological characteristics were recorded at least 35 times for each feature and documented using an Olympus BX-51 microscope. The macromorphology of the colonies was documented on MEA (Oxoid, Basingstoke, UK), potato dextrose agar (PDA; Himedia, Mumbai, India), and Sabouraud dextrose agar (SDA; Himedia, Mumbai, India) at 25, 30, and 37°C. The colonies were documented using a Canon EOS 500D camera. Color names were determined using the ISCC-NBS centroid color charts (87; hexadecimal color codes assigned independently according to the website are listed in the parentheses https://coolors.co).

Data availability.

The DNA sequences obtained in this study were deposited into the GenBank database (www.ncbi.nlm.nih.gov) under accession numbers MW936599 to MW936628, MW959139 to MW959143, MZ314457, MZ320340, MZ320335, and MZ320330. The alignments are available in the Dryad Digital Repository at https://doi.org/10.5061/dryad.59zw3r275. The names of newly described species were deposited in MycoBank (MB839323 and MB839324).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only. SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We declare no conflicts of interest.

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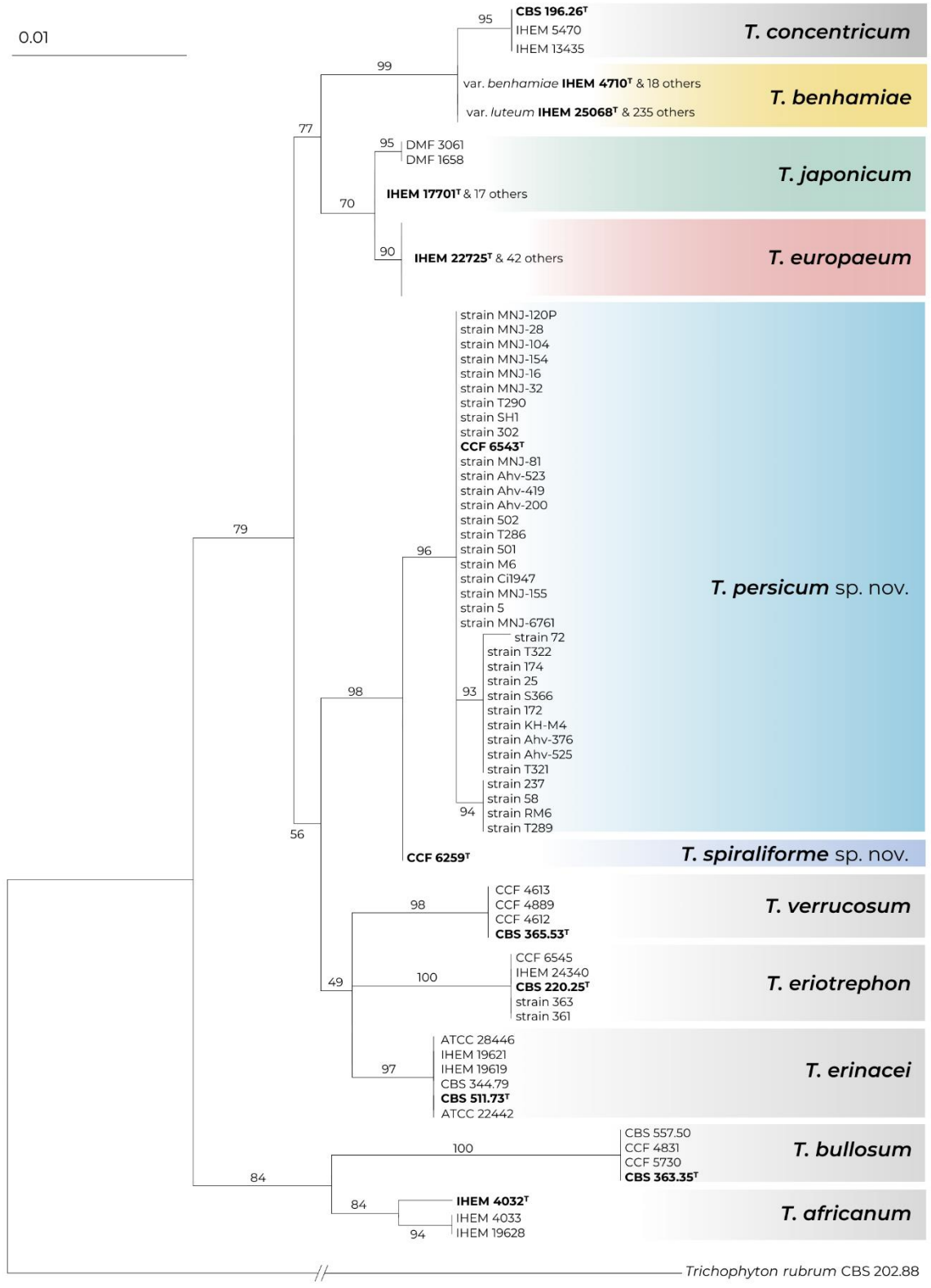
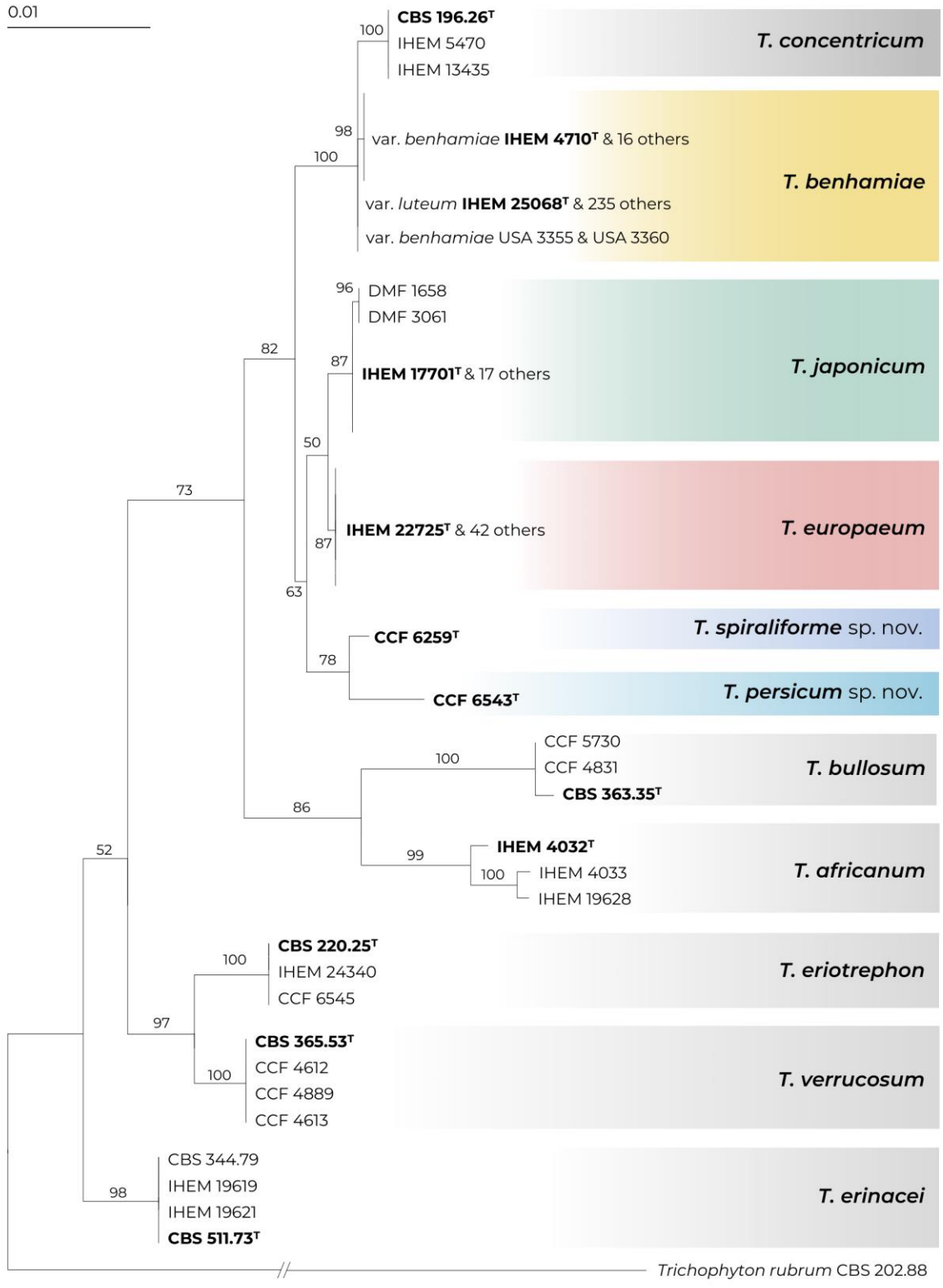
Additional supplementary material is available for download here::



Supplementary Figure S1. A best scoring Maximum Likelihood tree based on ITS rDNA region showing relationships of *Trichophyton spiraliforme* sp. nov. and *Trichophyton persicum* sp. nov. to other dermatophytes belonging to the *T. benhamiae* complex. Ex-type isolates are designated by a superscript T. *Trichophyton rubrum* CBS 202.88 was used as the outgroup.



Supplementary Figure S1. A best scoring Maximum Likelihood tree that includes only strains with complete sequence data available for four loci (*gapdh*, *tubb*, ITS rDNA and *tef1-α*). The tree shows the relationships of *Trichophyton spiraliforme* sp. nov. and *Trichophyton persicum* sp. nov. to other dermatophytes belonging to the *T. benhamiae* complex. Ex-type isolates are designated by a superscript T. *Trichophyton rubrum* CBS 202.88 was used as the outgroup.

Table S1. Species-specific conserved substitutions in the ITS rDNA region of *Trichophyton benhamiae* clade species[1]

Compared taxa	**Number of substitutions**	**Position of conserved substitution / indel** (positions refer to the alignments available in the Supplementary material)
T. benhamiae – T. concentricum	2	80 (C->T), 526 (T->C)
T. benhamiae – T. europaeum	6	41 (A->G), 43 (T->C), 54 (A->G), 266 (T->C), 471 (A->G), 475 (T->C); indel 609
T. benhamiae – T. japonicum	7	41 (A->G), 43 (T->C), 54 (A->G), 168 (T->C), 266 (T->C), 471 (A->G), 475 (T->C); indel 609
T. benhamiae – T. persicum	12	41 (A->G), 168 (T->C), 230 (G->A), 266 (T->C), 437 (C->T), 456 (A->G), 471 (A->G), 475 (T->C), 476 (C->T), 550 (G->A), 562 (C->T), 564 (C->T); indel 213
T. benhamiae – T. spiraliforme	10	41 (A->G), 168 (T->C), 230 (G->A), 266 (T->C), 471 (A->G), 475 (T->C), 476 (C->T), 550 (G->A), 562 (C->T), 564 (C->T); indel 213
T. concentricum – T. persicum	14	41 (A->G), 80 (T->C), 168 (T->C), 230 (G->A), 266 (T->C), 437 (C->T), 456 (A->G), 471 (A->G), 475 (T->C), 476 (C->T), 526 (C->T), 550 (G->A), 562 (C->T), 564 (C->T); indel 213
T. concentricum – T. europaeum	8	41 (A->G), 43 (T->C), 54 (A->G), 80 (T->C), 266 (T->C), 471 (A->G), 475 (T->C) & 526 (T->C); indel 609
T. concentricum – T. japonicum	9	41 (A->G), 43 (T->C), 54 (A->G), 80 (T->C), 168 (T->C), 266 (T->C), 471 (A->G), 475 (T->C) & 526 (C->T); indel 609
T. concetricum – T. spiraliforme	12	41 (A->G), 80 (T->C), 168 (T->C), 230 (G->A), 266 (T->C), 471 (A->G), 475 (T->C), 476 (C->T), 526 (C->T), 550 (G->A), 562 (C->T), 564 (C->T); indel 213
T. europaeum – T. persicum	10	43 (C->T), 54 (G->A), 168 (T->C), 230 (G->A), 437 (C->T), 456 (A->G), 476 (C->T), 550 (G->A), 562 (C->T), 564 (C->T); indel 213, indel 609
T. europaeum – T. spiraliforme	8	43 (C->T), 54 (G->A), 168 (T->C), 230 (G->A), 476 (C->T), 550 (G->A), 562 (C->T), 564 (C->T); indel 213, indel 609
T. japonicum – T. persicum	9	43 (C->T), 54 (G->A), 230 (G->A), 437 (C->T), 456 (A->G), 476 (C->T), 550 (G->A), 562 (C->T), 564 (C->T); indel 213, indel 609
T. japonicum – T. europaeum	1	168 (C->T)
T. japonicum – T. spiraliforme	7	43 (C->T), 54 (G->A), 230 (G->A), 476 (C->T), 550 (G->A), 562 (C->T), 564 (C->T); indel 213, indel 609
T. persicum – T. spiraliforme	2	437 (T->C), 456 (G->A)

[1] based on the ITS alignment deposited in the Dryad digital repository

PAPER 3

Surveillance of dermatophytosis due to Trichophyton erinacei in wild hedgehogs

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Detection and control of dermatophytosis in wild European hedgehogs (*Erinaceus europaeus*) admitted to a French Wildlife Rehabilitation Centre

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ABSTRACT

The rising number of European hedgehogs (*Erinaceus europaeus*) admitted every year to wildlife rehabilitation centres might be a source of concern to animal and public health since transmissible diseases, such as dermatophytosis, can be easily disseminated. This study seeks to evaluate the frequency of dermatophyte detection in hedgehogs admitted to a wildlife rehabilitation centre located near Paris, France, and to assess the risk of contamination in the centre in order to adapt prevention measures. A longitudinal cohort study was performed on 412 hedgehogs hosted at the Wildlife Animal Hospital of the Veterinary College of Alfort from January to December 2016. Animals were sampled once a month for fungal culture. Dermatophyte colonies were obtained from 174 out of 686 skin samples (25.4%). Besides *Trichophyton erinacei*, *Trichophyton mentagrophytes* and *Nannizzi gypsea* were also found. Dermatophyte detection seemed to be associated with the presence of skin lesions, while more than one-third of *T. erinacei*-positive animals were asymptomatic carriers. Healing required several months of treatment with topical and systemic azoles, but dermatophytosis did not seem to reduce the probability of release. Daily disinfection procedures and early detection and treatment of infected and asymptomatic carriers succeeded in limiting dermatophyte transmission between hedgehogs and humans.

KEYWORDS: Dermatophytosis; Hedgehog; Rescue centre; *Trichophyton*; Dermatophyte

INTRODUCTION

Trichophyton erinacei is a zoophilic dermatophyte transmitted from hedgehogs; it belongs to the *T. benhamiae* complex, along with eight other zoo- and anthropophilic pathogens [1]. Infection usually occurs by direct contact with an infected hedgehog, although indirect contact with contaminated material such as their nests has also been described [2,3]. While clinical lesions are not always visible in hedgehogs, the symptoms in humans are mostly depicted as inflammatory skin infections [2–4]. People at risk of infection are mainly owners of hedgehogs as exotic pets or handlers of these animals in wildlife rescue centres or animal hospitals [5,6]. The general public's participation in wildlife protection is steadily increasing by means of animal rescue in urban areas or volunteering in wildlife rescue centres [7]. Unfortunately, uninformed intervention in wildlife rescue rises questions concerning the risk of zoonotic disease transmission and the harmonisation of recommendations regarding wildlife handling.

The European hedgehog (*Erinaceus europaeus* Linnaeus, 1758) is a nocturnal insectivorous mammal with a wide distribution throughout western and central Europe [8]. In several regions of its range, the decline of the hedgehog population has been described [9,10]. In 1979, this situation led their inclusion in Appendix III of the Bern Convention on the Conservation of European Wildlife and Natural Habitats. In France, hedgehogs have been designated as a species of conservation concern since 1981 [11]. The presence of *T. erinacei* in European hedgehogs has already been reported [12,13], but, so far, information regarding its prevalence is based on a small dataset. On the other hand, the presence of hedgehog populations as urban dwellers can account for an adaptation to urban and suburban areas because of the loss of their habitat and food sources after urbanisation, agriculture intensification, and the use of pesticides [10,14,15]. Nevertheless, European hedgehogs in urban areas also face a decline in their population that can be associated with several causes, including road collisions [16]. The increasing interaction of hedgehogs with humans stresses the need to establish minimal health precautions to avoid contaminations [17].

In this study, we determine the frequency of dermatophyte detection in injured or orphaned European hedgehogs sheltered at the Wildlife Animal Hospital of the Veterinary College of Alfort, France (Chuv-FS Alfort), over a one-year period. Animals were sampled for the presence of dermatophytes by culture every month until release. Finally, an audit of the protocol for dermatophyte control allowed us to propose safety measures to improve recovery and release times and to avoid cross-contamination between hedgehogs or zoonotic transmission to rescuer workers.

MATERIALS AND METHODS

Bioethics

This study was carried out in accordance with the Article 214.90 of the French Rural Code and Directive 2010/63/EC of the European Parliament regarding the protection of animals used for experimental and other scientific purposes. Indeed, this study did not need previous approval by an ethics committee as it

did not include any experimental procedure likely to produce pain, suffering, distress or lasting harm equivalent to or higher than that caused by the introduction of a needle, in accordance with good veterinary practices.

Animals and Sampling

All hedgehogs arriving at Chuv-FS Alfort in 2016 were sampled and held complete information regarding origin, age, sex, date of arrival and weight (Supplementary Data Table S1). Before sampling, the animals were submitted to an extensive clinical examination, including the detection of ectoparasites and skin lesions suggesting dermatophyte infection, such as spine loss, crusty skin and erythema [18]. Clinical examination was performed under anaesthesia by isoflurane inhalation (Vetflurane® Virbac, Carros-France). The animals were sampled by scrubbing the whole skin surface with a 5-cm^2 autoclavesterilised carpet square [19]. Animals were resampled on a monthly basis until their release. Additionally, the skin scraping test was performed on animals with skin lesions by rubbing off a layer of cells until the capillary oozed with the edge of a blunt scalpel blade. Only dermatophyte-free animals were released.

During the winter season, all dermatophyte-negative hedgehogs arriving in November or later in good body condition (>450 g) were put on hibernation until March in individual boxes located in an isolated shelter. Bedding composed of straw and wood chips, dry food and water were provided when needed, and visual inspections of cages were scheduled twice a week.

Treatment Protocols

Animals with skin lesions suggesting dermatophytosis were treated for one month, four times in a three-day interval, with enilconazole (Imaveral® Audevard, Clichy, France). When dermatophyte colonies were detected by culture, treatment with itraconazole at 10 mg/kg s.i.d (Itrafungol® Elanco, Cuxhaven, Germany) was added to the enilconazole protocol. This treatment consisted of three cycles of one-week treatment with a one-week interval between treatments. Cages and all reusable material in contact with the hedgehogs were washed and disinfected on a daily basis. All materials were cleaned with or immersed in sodium hypochlorite 4% for at least 15 min (La Croix® Colgate-Palmolive Company, Colombes, France).

Culture and Dermatophyte Identification

The skin-exposed carpet face was put in five-second contact with Sabouraud dextrose agar (SDA) plates complemented with 0.5 g/L of chloramphenicol (Merck KGaA, Darmstadt, Germany) and 0.5 g/L of cycloheximide (Actidione®, Merck KGaA, Darmstadt, Germany) and then incubated at 30 °C for up to 14 days. All fungal cultures were evaluated both macroscopically and microscopically in terms of colony growth, pigment production and the presence of key characteristics following the morphology keys previously described [20]. Eight selected isolates of *T. erinacei* were subjected to a detailed analysis, which involved the characterisation of micromorphological features and macromorphology on three

agar media, including malt extract agar (MEA, Himedia, Mumbai, India), potato dextrose agar (PDA, Himedia, Mumbai, India), and SDA [21] at 25 °C. The macromorphology of the colonies was documented using an Olympus SZ61 or Canon EOS 500D camera, and micromorphology was documented using an Olympus BX-51 microscope. The colour of the colonies was determinate using the ISCC-NBS centroid colour charts [22]. Selected isolates were deposited with the Culture Collection of Fungi (CCF), Department of Botany, Charles University, Prague, Czech Republic.

Confirmation of initial identification from a randomised sampling of 124 positive cultures was performed by sequencing the ITS rDNA region (ITS1-5.8S-ITS2 cluster). DNA was extracted from ten-day-old colonies using the ArchivePure DNA Yeast and Gram2+ Isolation Kit (5 PRIME Inc., Gaithersburg, MD, USA), according to the manufacturer's instructions, with some modifications [23]. The ITS rDNA region was amplified using the primer set ITS1F and ITS4 [24]. PCR product purification followed the protocol of Réblová et al. [25]. Automated sequencing was performed with Macrogen Sequencing Service (Amsterdam, The Netherlands) using both terminal primers. Editing of the PCR products sequences was performed using BioEdit Sequence Alignment Editor Software [26]. The sequences were compared with those derived from the ex-type and reference strains, which are deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) using the BLAST algorithm.

Furthermore, 16 dermatophyte isolates showing morphological features not compatible with *T. erinacei* were further analysed by mass spectrometry (MALDI-TOF MS). Protein extract samples were obtained, as previously recommended by L'Ollivier et al., for the dermatophytes [27], with slight modifications. Briefly, a small piece of mycelium was gently scraped from the culture plate with a scalpel and suspended in 900 µL absolute ethanol (ethyl alcohol anhydrous; Carlo Erba SDS, Val de Reuil, France) and 300 µL HPLC water (Water HPLC; Prolabo BDH, Fontenay-sous-Bois, France). The sample was vortexed and centrifuged at 13,000× *g* for 10 min, with the resulting pellet air-dried and resuspended in 12.5 µL of 70% formic acid (Sigma-Aldrich, Lyon, France). After 5 min incubation at room temperature, 12.5 µL of 100% acetonitrile (Prolabo BDH, Fontenay-sous-Bois, France) was added over 5 min at room temperature, and the sample was then centrifuged at 13,000× *g* for 2 min. One µL of supernatant was spotted in duplicate onto an MTP 96 target plate polished steel TF (Bruker Daltonics GmbH, Bremen, Germany) and then air-dried. Then, the spot was covered with 1 µL of matrix solution (alpha-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Lyon, France), saturated in 50:25:25 acetonitrile:HPLC water:10% TFA) and air-dried. A bacterial test standard (Bruker Daltonics) was used for instrument calibration. MALDI-TOF MS species identification of the spectra was performed using a Microflex LT/SH smart mass spectrometer (Bruker, Bremen, Germany) and the MSI online application (https://biological-mass-spectrometry-identification.com/msi/welcome) developed by Marseille's Teaching Hospital in collaboration with the BCCM/IHEM collection in Brussels [28].

Statistical Analysis

Animals were categorised into three groups according to their age and weaning status: hoglets (<200 g), juveniles (200–400 g) and adults (>400 g) [29]. Comparisons of dermatophyte detection with origin, sex, age-group, month of arrival, presence of skin lesions and animal outcome (released versus deceased) were made using a chi-square test or Fisher's exact test when appropriate. The presence of skin lesions and the number of colonies (less than 10 vs. 10 or more colonies) were also analysed using the chi-square test. The length of stay at the rehabilitation centre before release was compared to the detection of dermatophytes by log-rank (Mantel–Cox) test. The same statistical analysis was used to compare the healing time with the number of colonies. All data were analysed using Prism software (v.5, GraphPad software, San Diego, CA, USA). A p-value < 0.05 was considered significant. Microconidia and macroconidia sizes were expressed as a size range (mean ± standard deviation).

RESULTS

Out of the 462 wild hedgehogs rescued during 2016, 412 were included in the present study. The other 50 animals were excluded because three or more epidemiological variables were missing. When data such as age (n = 3), sex (n = 81) or origin (n = 38) were not available during clinical examination, the animal with the missing data was excluded from the corresponding epidemiological analysis.

The origin of animals was mainly from the Ile-de-France region (349/374; 93.3%), but some hedgehogs also came from neighbouring regions such as Centre-Val de Loire, Normandie, Hauts-de-France and Grande-Est (Figure 1). One hedgehog came from as far as the Pays-de-la-Loire region. The number of hedgehogs arriving at the centre fluctuates during the year (Figure 2). Few arrivals occur during the winter season (n = 10); later, the numbers increase with time, reaching a peak during the summer season. In 2016, 225 out of 412 hedgehogs arrived between June and August, and half of them were hoglets. In total, 167 of 412 animals were adults (40.5%), 80 out of 412 were juveniles (19.4%) and 162 out of 412 were hoglets (39.3%). The sex rate was homogeneous for the three age groups. During the clinical examination on arrival, we observed ectoparasites such as fleas (*Archaeopsylla erinacei*) and ticks (Ixodes sp.) on most animals, with variable degrees of infestation. The hedgehogs with the poorest body condition on arrival were found systematically infested by Calliphoridae (Diptera) eggs and/or larvae (responsible for cutaneous myiasis). Thirty-two out of 412 hedgehogs (8%) showed skin lesions such as scaly skin, loss of spines or alopecia, with different degrees of severity (Figure 3). Skin scrapings were performed on animals showing itching, crusty skin and erythema on arrival. The presence of mites (*Caparinia tripilis* and *Sarcoptes scabiei)* was observed in two animals, respectively.

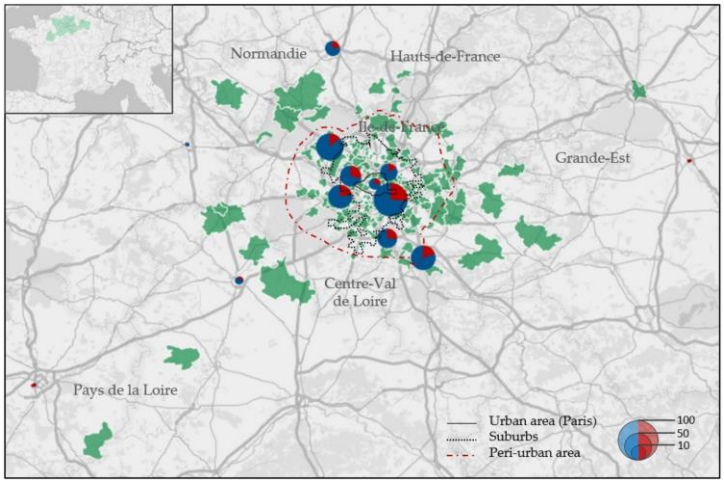

Fig. 1 Geographical origin of dermatophyte-negative (blue) and -positive (red) hedgehogs on their arrival at the rescue centre in 2016. Green-coloured regions correspond to departments (upper-left panel) or postal codes (main panel) from where hedgehogs were found. Laboratory results have been grouped by departments. Paris city is included. Size of the circles indicates the number of animals sampled. Administrative and landscaped divisions in Île de France have been adapted from the French Institute of Statistics and Economic Studies (INSEE).

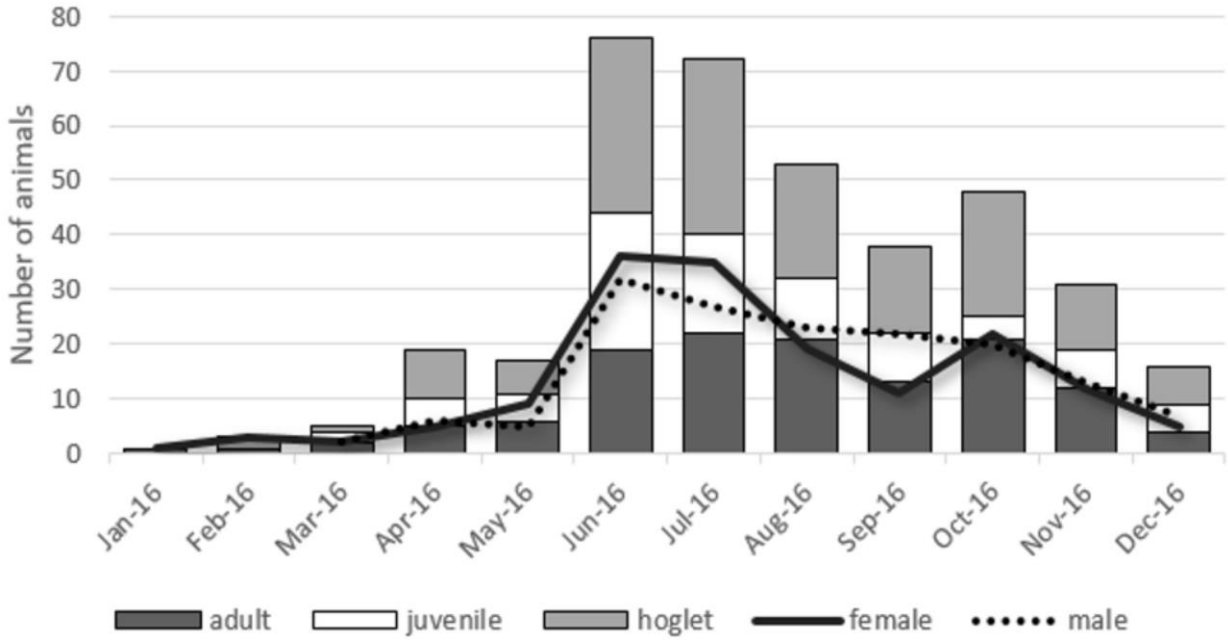

Fig. 2 Distribution of hedgehog arrivals over the year according to their age groups (bars) or sex (lines).

Dermatophyte Detection and Identification

Ninety-six out of 412 animals (23.3%) were diagnosed as infected on arrival (Table 1). Cultures revealed the presence of dermatophytes in 186 out of 726 samples (25.6%). *Trichophyton erinacei* was identified morphologically in 174 samples out of 726 (24%). A selection of 124 isolates was submitted for ITS sequencing. The ITS rDNA sequences of *T. erinacei* strains showed 100% similarity with the *T. erinacei*

ex-type strain CBS 511.73 (MN974540). Six isolates were identified as *T. mentagrophytes* and showed 99% similarity with the ex-neotype strain of *T. mentagrophytes* IHEM 4268 (MF926358) [30] and 100% similarity with the strain CBS 110.65 (MH858507). Further identification by mass spectrometry confirmed the identification of 16 isolates as *T. erinacei* (*n* = 10), *Nannizia gypsea* (*n* = 2), *T. interdigitale* (*n* = 2) or *A. quadrifidum* (*n* = 2).

A finely granular, white (#F2F3F4) to light-yellow (#F8DE7E) obverse and a paleyellow (#F3E5AB) to vivid orange-yellow (#F6A600) reverse characterized colonies of *T. erinacei* on SDA (Figure 4A,B). A coarsely granular, white (#F2F3F4) to yellowish-white (#F0EAD6) obverse and a pale-yellow (#F3E5AB) to vivid orange-yellow (#F6A600) reverse characterized colonies of *T. erinacei* on PDA (Figure 4C,D). A granular, white (#F2F3F4) obverse and a pale-yellow (#F3E5AB) to brilliant-yellow (#FADA5E) reverse characterised colonies of *T. erinacei* on MEA (Figure 4E,F). The colony diameter ranged from 24 to 29 mm (⌀= 27 mm) on SDA, from 19 to 23 mm (⌀= 21 mm) on PDA and from 19 to 27 mm (⌀= 26 mm) on MEA at 25 °C after 7 days. Clavate or pyriform microconidia 2.8–5.5 (4.0 ± 0.8) × 1.6–2.6 (2.1 ± 0.5) µm, borne on short conidiophores, were abundantly present in all samples. Macroconidia were rare, and they usually consisted of 2–4 cells (predominantly two-celled). No spiral hyphae were found even after 21 days of incubation.

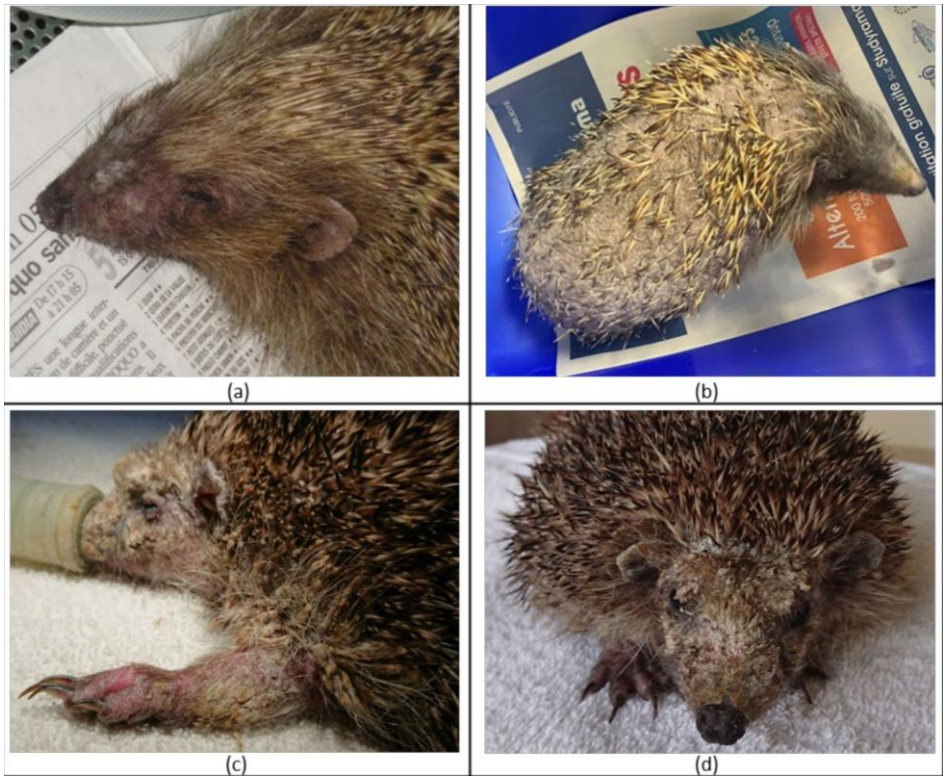

Fig. 3 Mild (**a**), moderate (**b**) and severe (**c**,**d**) skin lesions observed in wild European hedgehogs (*E. europaeus*) during clinical examination on admission to the rescue centre. Lesions are often associated with localised (**a**) or generalised (**c**,**d**) scaly skin, alopecia (hair loss), spine loss (**b**) and erythema (**c**).

Table 1. Distribution of culture results over monthly sampling of European hedgehogs admitted to the rescue centre (Chuv-FS Alfort). centre (Chuv-FS Alfort).

Sampling	*T. erinacei*		No Longer Followed (Released/Dead)	TOTAL
	Positive	Negative		
First	96	316	-	412
Second	44	90	278	412
Third	18	60	56	134
Fourth	9	27	42	78
Fifth	1	19	16	36
Sixth	1	4	15	20
Seventh	0	1	4	5
Total	169	517		

Morphological Characterisation of Dermatophytes

Isolates of *T. mentagrophytes* species displayed similar macromorphology to *T. erinacei* on SDA. The main diagnostic criteria to distinguish between these two species are colony reverse on SDA in shades of rusty deep-orange (#BE6516) to deep reddish-orange (#AA381E), coarsely granular colony texture on all examined media (MEA, SDA, PDA) (Figure 5) and the presence of spiral hyphae in most of the *T. mentagrophytes* isolates (Figure 6A). Conidia of *T. mentagrophytes* are predominantly globose to subglobose, while those of *T. erinacei* are predominantly clavate. However, some T. erinacei isolates may occasionally show granular colony texture and rusty deep-orange reverse on SDA [31]. Due to the high similarity between these two species and because some isolates did not develop characteristic features, identification was confirmed by sequencing and MALDI-TOF spectra data.

Epidemiological and Therapeutic Analysis

The global rate of dermatophyte-positive samples was 25.4% (174/686), with 169 out of 686 samples being *T. erinacei*-positive (24.6%). This corresponds to 123 out of 412 animals hosted in 2016. Chi-square tests of independence showed no association between the detection of dermatophytes and the origin, sex, age-group or month of arrival ($p > 0.05$). The same proportion of males (38/162; 23%) and females (41/169; 24%) were positive. Estimated trimestrial prevalence of dermatophyte infection or carriage remained at 20–30% during the year. Similar results were observed in hedgehogs classified by age: 39 out of 167 adults (23.4%), 17 out of 80 juveniles (21.3%) and 39 out of 162 hoglets (24.1%)

were positive for dermatophytes. Mean prevalence of positive hedgehogs among administrative departments with more than 10 animals was 22.7%, ranging from 13.6% (Val-d'Oise) to 28.6% (Hauts-de-Seine).

On arrival, 20 out of 32 animals with skin lesions (62.5%) were positive for *T. erinacei*. Among those without skin lesions, 76 out of 380 hedgehogs (20%) were culture-positive. After analysis, there was a significant relationship between these variables—animals with skin lesions were more likely to suffer from dermatophytosis (χ^2, $p = 0.0303$). Nevertheless, 76 out of 96 positive animals did not show skin lesions (79.2%). Cultures with less than ten colonies per plate were obtained in 55 out 96 of positive animals (57%). Only 20 out of 96 hedgehogs with cultures yielding 10 or more colonies per plate exhibited skin lesions (20.8%) and no statistical association between the number of colonies of dermatophyte and the presence of skin lesions was observed (χ^2, $p > 0.05$). The rate of positive animals during their first, second and third sampling was 23.3% (96/412), 32.8% (44/134) and 23.1% (18/78), respectively (Table 1). The contamination by *T. erinacei* at time of arrival was not associated with the outcome of the animals (released vs. deceased). On the other hand, the length of stay was significantly higher ($p < 0.0001$) for positive animals on arrival (mean length stay of 105 days) than for negative animals (mean length stay of 39 days). Finally, 16 hedgehogs with a negative fungal culture were put into hibernation. At the end of the hibernation period, they were sampled again, and 3 out of 16 animals were culture-positive, with 1–5 dermatophyte colonies per plate.

To propose proper safety measures, we screened 41 positive hedgehogs during their entire stay at the centre. Six asymptomatic animals became spontaneously negative one month after their arrival. The remaining 35 animals with skin lesions were further treated. After one month of topical enilconazole treatment, 18 animals (51.4%) became culturenegative or had fewer colonies in their cultures, with a remission of skin lesions. A Mantel– Cox log-rank test confirmed that animals with less than 10 colonies in culture got rid of the infection faster than those with 10 or more colonies ($p = 0.0016$). Following two-month combined therapy (enilconazole plus itraconazole treatment), the remaining 17 hedgehogs (48.6%) were either culture-negative or yielded less than 10 colonies. Among them, eight infected animals became negative for up to 4 months after arrival.

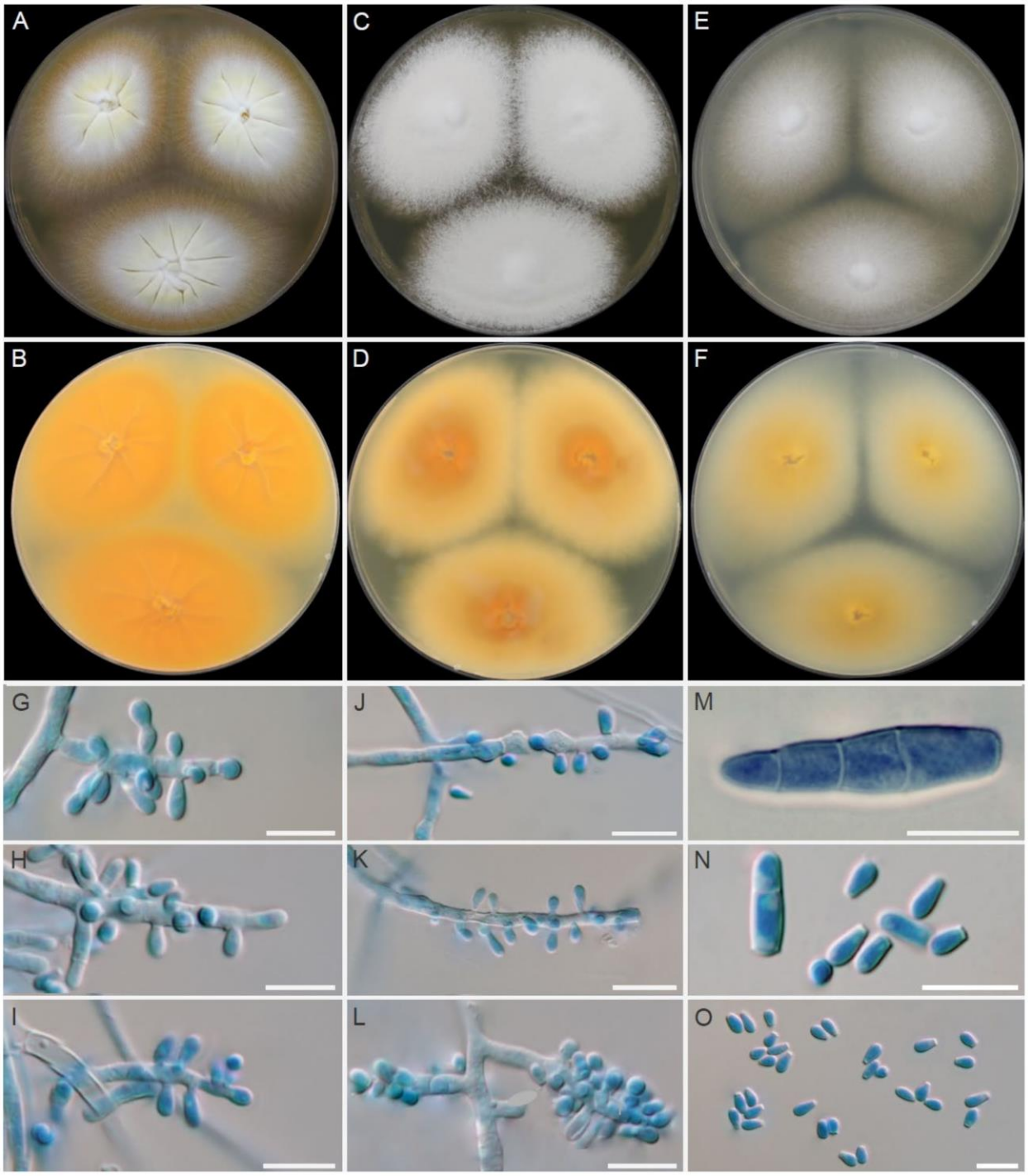

Fig. 4 Macromorphology and micromorphology of *Trichophyton erinacei*. Colonies after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**A**,**B**), potato dextrose agar (**C**,**D**) and malt extract agar (**E**,**F**). Conidiophores bearing microconidia (**G**–**L**); macroconidia (**M**); free microconidia and two-celled macroconidia (**N**); microconidia (**O**). Scale bars = 10 µm.

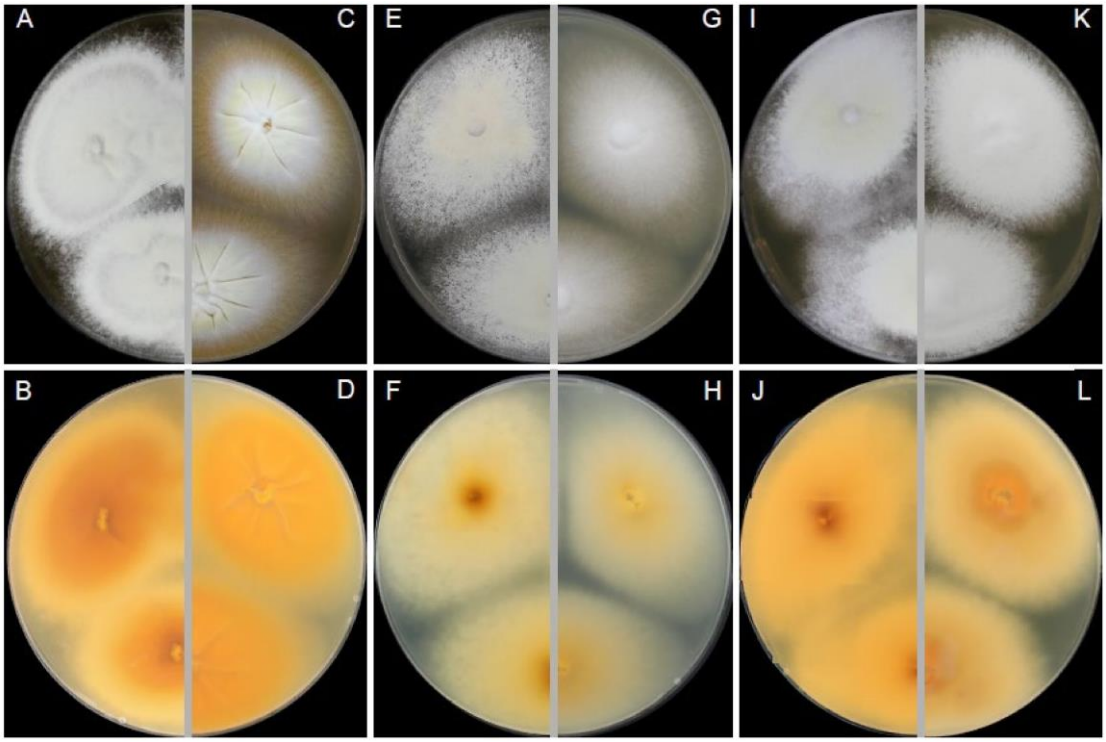

Fig. 5. Macromorphology of *Trichophyton mentagrophytes* (left) in comparison with *T. erinacei* (right) isolated from European hedgehogs in France. Colonies of *T. mentagrophytes* after two weeks of cultivation at 25 °C on Sabouraud's dextrose agar (**A**,**B**), malt extract agar (**E**,**F**) and potato dextrose agar (**I**,**J**). Colonies of *T. erinacei* under the same conditions on Sabouraud's dextrose agar (**C**,**D**), malt extract agar (**G**,**H**) and potato dextrose agar (**K**,**L**).

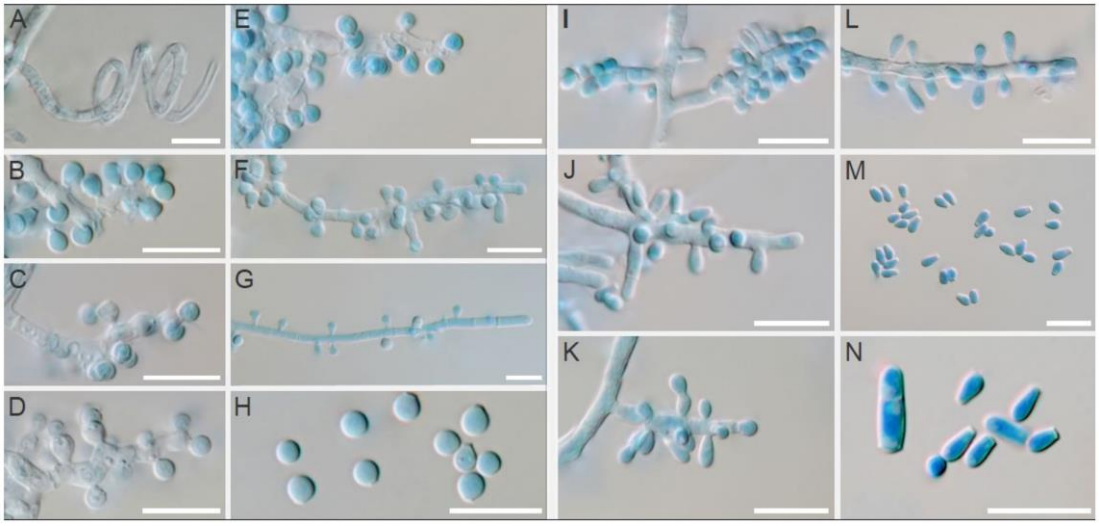

Fig. 6. Micromorphology of *Trichophyton mentagrophytes* (left; **A**–**H**) in comparison with *T. erinacei* (right; **I**–**N**) isolated from European hedgehogs in France. Spiral hyphae of *T. mentagrophytes* (**A**); branched or unbranched conidiophores bearing small round microconidia of *T. mentagrophytes* (**B**–**G**); free small round microconidia of *T. mentagrophytes* (**H**); simple conidiophores bearing microconidia of *T. erinacei* (**I**–**L**); free clavate microconidia and two-celled macroconidia of *T. erinacei* (**M**,**N**). Scale bars = 10 µm.

DISCUSSION

Wildlife care in rescue centres must include the use of appropriate measures to ensure the fast recovery of the hosted animals, avoiding the risk of transmission of infectious agents to other animals or to the caretakers. Our study confirmed that the dermatophyte species *T. erinacei* may be frequently detected in wild European hedgehogs, mostly coming from suburban areas in France, and that this contamination is often unnoticed. A higher prevalence (23.3%) was observed in comparison to a previous study focused on free-ranging European hedgehogs (13%) or captive European hedgehogs (21%) in France [13]. Differences in data may be linked to the inherent causes for their arrival to the wildlife rescue centre (e.g., diseased or injured animals, abandoned hoglets, undernourished animals), which could facilitate dermatophyte infection. High infection rates have been described in urban wild populations in Great Britain and New Zealand, ranging between 20–25% and 44.7%, respectively [12,32].

Previous studies already described no association between the presence of dermatophytes and the observation of evocative skin lesions [12]. The high number of positive cultures coming from asymptomatic hedgehogs (79.2%) confirm that in the context of wildlife rescue, proper measures such as the use of protective gloves and continuous environmental disinfection become crucial to avoid contagion. The monthly survey allowed us to establish close surveillance of the animals with a positive culture, and we could confirm the risk of contamination of the environment and of transmission to other hedgehogs or to handlers [17,33,34]. Previously, Bexton and Nelson [18] highlighted the importance of establishing adequate therapeutic protocols. During our study, topical treatment alone was not enough to cure dermatophyte infection. Indeed, 45.8% of treated animals (44/96) were still infected even after one month of enilconazole treatment. Only combined therapy, i.e., combined topical and oral azole treatment allowed the elimination of the dermatophyte or, at least, a decrease of fungal load in almost all animals (394/412; 95.6%) after up to three months of treatment.

The head of the hedgehogs has been previously described as the most frequent site of infection, while thorough sampling of the animal is also advised since other sites can also be infected [12,35]. We decided to sample animals under isoflurane anaesthesia using the carpet technique, which reduces the risk of underestimating the infection rates [12]. Males and young individuals have been previously described as being more susceptible to dermatophyte infection [12,18]. However, the present study did not reveal any association between fungal load at culture and sex or age. This cannot be explained by the fact that most of the positive animals were asymptomatic, while previous studies were focused on hedgehogs with skin lesions [12,36,37]. Most likely, there would be heterogeneity among the studies to determine the age of hedgehogs, which can only be accurately estimated with a postmortem histological analysis of the jaw [38]. The fact that the prevalence of dermatophytes was not associated with a specific season suggests that environmental conditions may not have a major role in transmission. Nevertheless, a larger number of animals sampled during winter would be needed to confirm this observation, since the risk of contamination through the use of contaminated nests during hibernation has been already described [39]. Finally, the concomitant presence of mites was observed in only two animals of the

present study and, as a consequence, it was not possible to confirm the hypothesis that mites may enhance *T. erinacei* transmission, as previously reported by English and Morris [39].

Nest contamination during hibernation could also be an explanation for the three hedgehogs that were highly contaminated at the time of their arrival. They needed three to four months of itraconazole treatment before dermatophyte withdrawal in order to be allowed to hibernate, and they showed a relapse after hibernation. Bexton and Nelson (2016) reported lower cure rates with itraconazole than with terbinafine [18], while recurrence has been described after long-term antifungal treatment with antifungal drugs such as terbinafine [40]. In the present study, the relapse might have happened because the culture technique was not sensitive enough to detect a residual presence of dermatophytes before hibernation, while the straw used as bedding material could have participated in the conservation of remaining dermatophyte spores during the hibernation period. The detection of 21 hedgehogs that were negative at the time of their arrival, becoming positive one month later, seems to support this hypothesis. Further follow-up showed that 16 animals were negative again after one month of local treatment.

Even though the hedgehog-specific dermatophyte *T. erinacei* was the most frequent dermatophyte observed (94.6%), identification by mass spectrometry and sequencing allowed us to confirm the morphological examination as some isolates did not develop characteristic features. Additionally, *T. erinacei* morphology is relatively similar to the second most frequently observed species, *T. mentagrophytes*. While the host spectrum of *T. erinacei* is narrow (Erinaceinae subfamily), *T. mentagrophytes* has been reported from a broad spectrum of domestic animals. However, its host spectrum in wild animals remains poorly known, partly due to extensive taxonomic rearrangements in the past decades [41].

The presence of geophilic dermatophytes *Nannizia gypsea* and *T. terrestre* (syn. *Arthroderma quadrifidum*) in asymptomatic carriers was also reported. These results become of utmost importance if we consider the role of wild animals as carriers of dermatophytes and related fungi [42].

Finally, further research is needed to elucidate whether asymptomatic carriage is due to simple mechanical transport or to infection with isolates with lower virulence, as already suggested for *Microsporum canis* in cats [43]. Low virulence would be associated with decreased keratinolytic activity [44]. The presence of concomitant diseases in dermatophyte-infected hedgehogs does not seem to reduce the probability of release since the rate of positive hedgehogs at time of arrival was higher amongst the animals that were finally released (47/96; 49%) than amongst those that died at the centre (98/316; 31%). A comparative analysis of keratinase and elastase production in *T. erinacei* isolates would shed light on the differential pathogenic risk for hedgehogs and humans.

The high prevalence of asymptomatic carriers detected in this study stresses the risk of dermatophyte dissemination in rescued animals or zoonotic transmission to caretakers. Even if no human contamination was reported during this study, one caretaker and one clinician developed ringworm-associated lesions after the manipulation of animals without protective measures two years earlier.

Thanks to the detailed screening of infected animals during their treatment, the study brings up relevant suggestions, such as individual confinement when possible and daily disinfection of cages and tools in contact with the hedgehogs. These measures can be easily be adopted at rescue centres and will ensure the successful treatment of infected.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, as it did not include any experimental procedure likely to produce pain, suffering, distress or lasting harm equivalent to or higher than that caused by the introduction of a needle, in accordance with the Article 214.90 of the French Rural Code and Directive 2010/63/EC of the European Parliament regarding the protection of animals used for experimental and other scientific purposes.

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Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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Table S1: European hedgehogs (*Erinaceus europeaeus*) admitted at the rescue centre (ChuvFS Alfort) during 2016 and sampled for dermatophyte detection.

N°	ID	Date of arrival	Postal code	Outcome	Weight at arrival (g)	Sex	Skin lesions	1st sampling date	Sample ID	1st diagnosis	N° of samplings
1	**40**	17/01/2016	93160	R	550	female	no	17/01/2016	T256	negative	1
2	**64**	12/07/2016	77400	D	NA	NA	no	25/01/2016	T224	negative	1
3	**86**	13/07/2016	60940	R	154	female	no	01/02/2016	T254	negative	1
4	**122**	13/07/2016	60940	R	177	female	no	13/02/2016	T255	negative	1
5	**123**	13/07/2016	78340	D	585	female	no	13/02/2016	T258	positive	2
6	**186**	03/09/2016	NA	R	145	male	no	04/03/2016	T311	negative	2
7	**216**	03/09/2016	NA	R	315	male	no	11/03/2016	T412	positive	1
8	**281**	01/02/2016	94800	D	319	female	no	25/03/2016	T413	positive	3
9	**296**	01/05/2016	95260	R	1711	NA	yes	29/03/2016	T450	negative	2
10	**300**	01/07/2016	77185	D	540	female	no	30/03/2016	T449	negative	2
11	**319**	01/09/2016	94000	D	133	female	no	03/04/2016	T446	negative	1
12	**320**	01/12/2016	78640	E	291	female	no	03/04/2016	T447	negative	1
13	**322**	02/05/2016	95100	D	511	male	no	03/04/2016	T445	negative	1
14	**324**	02/06/2016	92700	D	402	male	no	04/04/2016	T444	negative	2
15	**364**	02/07/2016	78840	R	68	NA	no	08/04/2016	T493	negative	1
16	**388**	02/07/2016	78840	D	70	NA	no	12/04/2016	T494	negative	1
17	**390**	02/07/2016	95640	D	111	NA	no	12/04/2016	T508	negative	1
18	**393**	02/07/2016	95640	D	112	NA	no	13/04/2016	T507	negative	1
19	**403**	02/07/2016	95640	R	127	male	no	15/04/2016	T519	negative	2
20	**404**	02/07/2016	93160	D	156	NA	no	15/04/2016	TS20	negative	2
21	**415**	02/07/2016	93360	R	629	female	no	17/04/2016	T521	negative	1
22	**418**	02/08/2016	75019	D	477	female	no	18/04/2016	T576	positive	1
23	**427**	02/09/2016	77163	D	193	male	no	19/04/2016	T572	negative	1
24	**441**	02/09/2016	77860	E	NA	NA	no	22/04/2016	T574	negative	2
25	**446**	02/10/2016	78500	NA	154	NA	no	23/04/2016	T573	negative	2
26	**454**	02/10/2016	94170	D	373	NA	no	25/04/2016	T575	positive	3
27	**472**	02/11/2016	94170	D	330	male	no	26/04/2016	T612	negative	1
28	**489**	02/12/2016	NA	D	300	female	no	28/04/2016	T613	positive	2
29	**501**	03/03/2016	91400	R	651	male	no	29/04/2016	T614	positive	1

30	**503**	03/04/2016	77680	D	260	NA	no	29/04/2016	T615	positive	1
31	**530**	03/04/2016	94190	R	472	NA	no	01/05/2016	T618	positive	2
32	**533**	03/04/2016	94800	D	758	female	no	02/05/2016	T620	negative	1
33	**555**	03/05/2016	94260	D	387	female	no	03/05/2016	T634	negative	1
34	**582**	03/06/2016	94410	E	NA	NA	no	06/05/2016	T635	negative	1
35	**606**	03/07/2016	95570	D	73	NA	no	08/05/2016	T657	negative	1
36	**616**	03/07/2016	NA	R	119	male	no	08/05/2016	T656	negative	1
37	**656**	03/07/2016	91300	D	202	NA	no	12/05/2016	T736	positive	2
38	**671**	03/08/2016	92170	R	203	female	no	13/05/2016	T733	negative	2
39	**675**	03/08/2016	91330	E	720	female	no	14/05/2016	T739	negative	1
40	**715**	03/08/2016	91330	E	NA	female	no	15/05/2016	T735	negative	1
41	**763**	03/09/2016	94000	NA	116	female	no	17/05/2016	T742	negative	1
42	**766**	03/09/2016	77600	NA	143	NA	no	18/05/2016	T745	negative	1
43	**769**	03/09/2016	94500	D	190	female	no	18/05/2016	T741	negative	1
44	**818**	03/09/2016	92320	R	722	male	no	21/05/2016	T732	negative	2
45	**832**	03/10/2016	94130	NA	157	female	yes	22/05/2016	T747	negative	1
46	**873**	03/11/2016	NA	D	juvenile	male	no	24/05/2016	T804	positive	4
47	**894**	03/12/2016	94350	D	272	male	no	25/05/2016	T807	positive	1
48	**926**	03/12/2016	77560	NA	816	female	no	28/05/2016	T803	negative	1
49	**987**	04/04/2016	94360	R	1089	male	no	31/05/2016	T800	negative	1
50	**1017**	04/06/2016	94370	D	565	male	no	03/06/2016	T798	negative	2
51	**1048**	04/06/2016	92160	NA	835	male	no	03/06/2016	T799	negative	1
52	**1067**	04/07/2016	78260	D	128	male	no	04/06/2016	T806	negative	7
53	**1069**	04/08/2016	94500	R	175	male	no	04/06/2016	T797	negative	1
54	**1092**	04/09/2016	NA	D	310	NA	no	06/06/2016	T823	negative	5
55	**1093**	04/10/2016	77220	D	324	male	no	06/06/2016	T825	negative	1
56	**1107**	04/11/2016	95180	D	188	female	no	07/06/2016	T824	negative	5
57	**1117**	04/11/2016	78960	D	272	female	no	07/06/2016	T826	negative	1
58	**1131**	04/11/2016	78960	NA	325	male	no	08/06/2016	T827	negative	6
59	**1141**	04/11/2016	94130	NA	541	female	yes	08/06/2016	T829	negative	1
60	**1163**	04/12/2016	94170	D	504	female	yes	09/06/2016	T830	positive	1
61	**1203**	05/08/2016	78130	R	57	male	no	11/06/2016	T865	positive	7

62	**1209**	05/08/2016	93130	E	70	male	no	11/06/2016	T867	negative	5
63	**1210**	05/08/2016	93130	D	75	male	no	13/06/2016	T868	negative	5
64	**1211**	05/09/2016	78670	E	503	male	no	13/06/2016	1864	positive	5
65	**1212**	05/09/2016	92160	R	703	male	yes	11/06/2016	1866	negative	1
66	**1235**	05/10/2016	78180	D	74	female	no	13/06/2016	T863	positive	5
67	**1268**	06/05/2016	28100	D	600	female	no	13/06/2016	T861	negative	1
68	**1269**	06/06/2016	92230	R	212	NA	no	13/06/2016	T859	negative	1
69	**1270**	06/06/2016	75018	D	NA	NA	no	13/06/2016	T860	negative	1
70	**1273**	06/07/2016	94300	D	123	female	no	14/06/2016	T882	negative	1
71	**1281**	06/07/2016	78500	D	196	NA	yes	15/06/2016	T881	negative	1
72	**1313**	06/07/2016	94240	D	200	female	no	13/06/2016	T899	negative	2
73	**1320**	06/07/2016	94350	D	276	NA	no	18/06/2016	T898	negative	2
74	**1341**	06/07/2016	94500	R	668	NA	no	19/06/2016	T900	negative	1
75	**1347**	06/08/2016	92220	R	110	female	no	20/06/2016	T906	negative	2
76	**1348**	06/08/2016	94210	E	872	female	yes	19/06/2016	T897	positive	1
77	**1349**	06/09/2016	91190	D	130	female	no	19/06/2016	T901-1	positive	5
78	**1352**	06/09/2016	93110	E	NA	male	no	19/06/2016	T896	negative	1
79	**1354**	06/10/2016	92230	D	194	female	no	19/06/2016	T908	negative	1
80	**1362**	06/11/2016	77380	NA	224	female	no	19/06/2016	T901-2	negative	2
81	**1363**	06/11/2016	77100	R	406	male	no	19/06/2016	T901-3	negative	3
82	**1364**	06/12/2016	92160	NA	302	female	no	20/06/2016	T910	positive	1
83	**1376**	06/12/2016	91460	D	467	NA	no	20/06/2016	T911	negative	1
84	**1390**	06/12/2016	28130	E	juvenile	female	no	21/06/2016	T909	negative	4
85	**1405**	07/06/2016	77400	D	51	NA	no	22/06/2016	T925	negative	1
86	**1406**	07/06/2016	NA	R	104	female	no	22/06/2016	T926	negative	1
87	**1410**	07/07/2016	94380	D	80	NA	no	06/07/2016	T932	negative	2
88	**1431**	07/07/2016	78670	D	363	NA	no	23/06/2016	T929	negative	1
89	**1432**	07/07/2016	78960	R	365	female	no	23/06/2016	T930	negative	1
90	**1438**	07/07/2016	92340	D	471	NA	no	23/06/2016	T928	positive	5
91	**1449**	07/10/2016	95880	NA	315	female	no	23/06/2016	T927	negative	1
92	**1454**	07/11/2016	91179	D	juvenile	male	yes	24/06/2016	T955	negative	4
93	**1459**	08/04/2016	92210	D	1000	male	no	24/06/2016	T953	positive	5

94	**1460**	08/05/2016	93440	D	647	male	no	24/06/2016	T952	positive	5
95	**1461**	08/05/2016	NA	E	720	male	no	24/06/2016	T951	positive	5
96	**1466**	08/06/2016	92210	D	91	NA	no	24/06/2016	T954	negative	1
97	**1471**	08/06/2016	95470	NA	119	NA	no	24/06/2016	T948	negative	3
98	**1472**	08/07/2016	91200	R	90	female	no	24/06/2016	T949	negative	3
99	**1473**	08/07/2016	91200	D	98	male	no	24/06/2016	T950	positive	5
100	**1480**	08/08/2016	NA	D	28	female	no	24/06/2016	T947	negative	1
101	**1485**	08/08/2016	95240	D	127	male	no	24/06/2016	T946	negative	1
102	**1492**	08/08/2016	95240	R	135	female	no	25/06/2016	T956	negative	3
103	**1493**	08/08/2016	95240	E	146	male	no	25/06/2016	T957	negative	1
104	**1494**	08/09/2016	94700	D	257	male	no	25/06/2016	T958	negative	1
105	**1497**	08/11/2017	94000	D	208	female	no	25/06/2016	T959	negative	1
106	**1502**	09/06/2016	94260	D	NA	NA	no	26/06/2016	T960	negative	3
107	**1505**	09/07/2016	93100	D	70	female	no	26/06/2016	T961	negative	1
108	**1506**	09/07/2016	95580	D	77	female	no	26/06/2016	T962	negative	3
109	**1510**	09/07/2016	95580	D	79	male	no	26/06/2016	T963	positive	5
110	**1512**	09/07/2016	92410	D	200	female	no	26/06/2016	T964	positive	1
111	**1516**	09/07/2016	NA	R	308	male	no	26/06/2016	T967	positive	3
112	**1517**	09/07/2016	93100	E	529	female	no	26/06/2016	T966	negative	1
113	**1530**	09/09/2016	NA	D	60	female	no	26/06/2016	T968	negative	1
114	**1531**	09/09/2016	92000	D	NA	male	no	26/06/2016	T965	negative	1
115	**1532**	09/10/2016	92330	NA	135	male	no	26/06/2016	T979	negative	2
116	**1537**	09/10/2016	77181	D	217	male	no	27/06/2016	T986	positive	1
117	**1542**	09/10/2016	78150	E	530	male	no	27/06/2016	T984	positive	1
118	**1543**	09/10/2016	94420	E	NA	female	no	27/06/2016	T983	negative	2
119	**1544**	09/10/2016	91600	E	NA	male	no	27/06/2016	T982	negative	1
120	**1545**	09/12/2016	NA	D	242	female	no	27/06/2016	T981	positive	2
121	**1546**	09/12/2016	NA	D	288	female	no	27/06/2016	T980	positive	4
122	**1548**	09/12/2016	NA	D	331	female	no	27/06/2016	T985	negative	1
123	**1589**	10/07/2016	95880	D	58	male	no	29/06/2016	T978	positive	1
124	**1590**	10/07/2016	95880	D	64	female	no	29/06/2016	T1027	negative	1
125	**1596**	10/08/2016	78370	D	117	female	no	29/06/2016	T1021	negative	1

126	**1597**	10/08/2016	78370	D	130	male	yes	29/06/2016	T1020	negative	1
127	**1599**	10/08/2016	78370	R	888	male	no	30/06/2016	T1025	negative	1
128	**1600**	10/09/2016	92110	E	1000	female	no	30/06/2016	T1024	negative	1
129	**1601**	10/10/2016	94130	NA	266	male	yes	30/06/2016	T1023	negative	1
130	**1602**	10/10/2016	94130	D	298	female	yes	30/06/2016	T1022	negative	1
131	**1606**	10/10/2016	94130	NA	303	female	yes	30/06/2016	T1019	negative	1
132	**1624**	10/10/2016	94130	R	420	male	no	01/07/2016	T1026	positive	1
133	**1640**	10/12/2016	94360	D	339	male	no	01/07/2016	T1017	negative	1
134	**1658**	10/12/2016	91160	NA	459	female	no	02/07/2016	T1016	negative	1
135	**1659**	11/03/2016	78960	D	689	male	yes	02/07/2016	T1015	negative	1
136	**1660**	11/06/2016	75015	NA	105	male	no	02/07/2016	T1014	negative	3
137	**1664**	11/06/2016	95230	R	120	male	no	02/07/2017	T1013	negative	2
138	**1678**	11/06/2016	NA	R	121	female	no	02/07/2016	T1012	negative	1
139	**1680**	11/06/2016	95230	R	125	female	no	02/07/2016	T1010	negative	3
140	**1681**	11/06/2016	92160	D	387	female	no	02/07/2016	T1011	negative	1
141	**1688**	11/07/2016	95100	D	73	female	no	03/07/2016	T1009	negative	3
142	**1696**	11/07/2016	77590	D	juvenile	female	no	03/07/2016	T1029	negative	3
143	**1702**	11/07/2016	94370	D	NA	female	no	03/07/2016	T1008	negative	1
144	**1708**	11/09/2016	94120	NA	142	female	no	04/07/2016	T1038	positive	1
145	**1754**	11/09/2016	94120	D	NA	NA	no	07/07/2016	T1074	positive	1
146	**1761**	11/10/2016	95130	D	127	female	no	06/07/2016	T1078	positive	1
147	**1762**	11/10/2016	94290	D	NA	NA	no	06/07/2016	T1076	positive	1
148	**1765**	11/11/2016	60230	D	120	NA	no	06/07/2016	T1077	negative	1
149	**1775**	11/12/2016	77450	NA	470	female	no	06/07/2016	T1069	negative	1
150	**1780**	12/04/2016	93160	D	524	male	no	07/07/2016	T1080	negative	1
151	**1782**	12/04/2016	51100	E	NA	female	no	07/07/2016	T1072	positive	1
152	**1793**	12/05/2016	75005	E	557	NA	yes	07/07/2016	T1092	positive	1
153	**1795**	12/06/2016	NA	R	121	male	no	07/07/2016	T1081	negative	1
154	**1809**	12/07/2016	91160	E	700	NA	no	08/07/2016	T1071	positive	1
155	**1810**	12/07/2016	75017	R	1020	male	no	08/07/2016	T1062	negative	3
156	**1819**	12/07/2016	92220	D	NA	NA	no	09/07/2016	T1093	negative	1
157	**1820**	12/08/2016	NA	R	127	male	no	09/07/2016	T1095	positive	1

158	**1821**	12/08/2016	77380	E	NA	female	no	09/07/2016	T1094	positive	1
159	**1828**	12/09/2016	94400	E	110	NA	no	09/07/2016	T1096	negative	1
160	**1829**	12/09/2016	94400	NA	116	NA	no	09/07/2016	T1082	negative	1
161	**1832**	12/09/2016	94400	D	121	female	no	09/07/2016	T1067	negative	2
162	**1850**	12/10/2016	77181	D	223	male	no	10/07/2016	T1097	negative	1
163	**1851**	12/10/2016	94800	NA	230	male	no	10/07/2016	T1068	positive	1
164	**1865**	12/10/2016	77120	NA	274	NA	no	10/07/2016	T1083	positive	1
165	**1893**	12/10/2016	95000	D	284	female	yes	11/07/2016	T1099	negative	1
166	**1894**	12/10/2016	NA	D	NA	NA	no	11/07/2016	T1101	negative	1
167	**1910**	12/11/2016	78330	NA	377	NA	no	12/07/2016	T1064	negative	2
168	**1914**	12/11/2016	60500	D	406	female	no	13/07/2016	T1066	positive	1
169	**1915**	13/02/2016	75018	R	697	female	no	13/07/2016	T1100	negative	2
170	**1916**	13/02/2016	94140	R	1001	male	no	13/07/2016	T1065	negative	2
171	**1917**	13/04/2016	94350	D	503	male	no	13/07/2016	T1079	negative	1
172	**1918**	13/05/2016	94100	R	830	male	no	13/07/2016	T1063	positive	1
173	**1927**	13/06/2016	92410	D	82	male	no	14/07/2016	T1139	positive	1
174	**1941**	13/06/2016	78100	D	150	NA	no	14/07/2016	T1138	negative	1
175	**1945**	13/06/2016	78100	D	172	male	no	14/07/2016	T1134	negative	1
176	**1946**	13/06/2016	75012	R	313	male	no	14/07/2016	T1137	negative	1
177	**1947**	13/08/2016	95230	D	100	female	no	14/07/2016	T1136	negative	1
178	**1951**	13/08/2016	95230	R	108	NA	no	15/07/2016	T1135	negative	2
179	**1954**	13/08/2016	95230	D	126	male	no	15/07/2016	T1130	positive	2
180	**1955**	13/08/2016	95230	D	130	female	no	15/07/2016	T1131	negative	2
181	**1957**	13/08/2016	94440	R	350	female	no	15/07/2016	T1133	negative	2
182	**1958**	13/08/2016	77200	D	440	NA	no	15/07/2016	T1129	negative	1
183	**1962**	13/08/2016	NA	D	NA	NA	yes	16/07/2016	T1122	negative	1
184	**1990**	13/10/2016	75012	D	210	male	no	17/07/2016	T1126	negative	3
185	**1991**	13/10/2016	NA	D	313	NA	no	17/07/2016	T1132	negative	1
186	**2003**	13/11/2016	91080	D	506	female	no	17/07/2016	T1128	negative	1
187	**2011**	14/05/2016	94700	D	440	female	no	17/07/2016	T1127	negative	1
188	**2014**	14/06/2016	77090	D	109	female	yes	18/07/2016	T1125	negative	1
189	**2020**	14/07/2016	95100	D	120	female	no	18/07/2016	T1124	negative	1

190	**2022**	14/07/2016	94700	D	385	female	no	18/07/2016	T1123	negative	1
191	**2074**	14/07/2016	94700	D	483	male	no	20/07/2016	T1160	negative	2
192	**2085**	14/07/2016	77370	D	NA	NA	no	20/07/2016	T1161	negative	1
193	**2096**	14/09/2016	93420	D	99	female	no	21/07/2016	T1158	negative	1
194	**2106**	14/09/2016	93420	NA	103	female	no	21/07/2016	T1159	negative	1
195	**2111**	14/09/2016	93420	NA	103	female	no	21/07/2016	T1157	positive	1
196	**2158**	14/10/2016	77170	D	187	male	no	25/07/2016	T1188	negative	2
197	**2166**	15/04/2016	77340	R	854	female	no	25/07/2016	T1190	positive	4
198	**2169**	15/04/2016	77340	R	1076	male	no	25/07/2016	T1189	negative	2
199	**2171**	15/05/2016	NA	D	470	male	no	25/07/2016	T1185	negative	2
200	**2173**	15/06/2016	94360	D	335	NA	no	26/07/2016	T1186	negative	1
201	**2174**	15/07/2016	95530	R	130	female	no	26/07/2016	T1187	negative	1
202	**2181**	15/07/2016	94430	R	221	female	no	25/07/2016	T1184	positive	4
203	**2201**	15/07/2016	92160	R	680	female	no	26/07/2016	T1183	negative	1
204	**2207**	15/07/2016	77170	R	953	female	no	27/07/2016	T1182	negative	1
205	**2220**	15/07/2016	78670	D	NA	female	no	27/07/2016	T1180	negative	1
206	**2221**	15/08/2016	77310	E	10	NA	yes	27/07/2016	T1181	negative	1
207	**2222**	15/08/2016	77310	E	14	male	yes	27/07/2016	T1179	negative	1
208	**2225**	15/08/2016	77310	E	15	female	no	27/07/2016	T1177	negative	2
209	**2236**	15/09/2016	94440	D	340	male	no	28/07/2016	T1178	positive	1
210	**2269**	15/11/2016	78800	D	421	female	no	01/08/2016	T1229	negative	3
211	**2270**	15/12/2016	78340	NA	330	male	no	01/08/2016	T1228	negative	3
212	**2271**	16/07/2016	91430	D	528	male	no	01/08/2016	T1214	negative	3
213	**2272**	16/08/2016	77750	E	NA	NA	no	30/07/2016	T1260	negative	4
214	**2274**	16/09/2016	60100	D	173	NA	no	30/07/2016	T1221	negative	1
215	**2295**	16/10/2016	94000	D	178	male	no	31/07/2016	T1223	negative	2
216	**2301**	16/10/2016	77610	D	218	female	no	31/07/2016	T1224	Negative	1
217	**2308**	16/10/2016	NA	D	297	male	no	31/07/2016	T1225	positive	1
218	**2336**	16/10/2016	NA	D	347	male	no	02/08/2016	T1261	negative	1
219	**2339**	16/11/2016	77181	D	253	male	yes	03/08/2016	T1263	negative	2
220	**2349**	16/11/2016	77700	D	278	male	yes	03/08/2016	T1252	negative	1
221	**2351**	16/11/2016	NA	NA	NA	NA	yes	03/08/2016	T1268	negative	2

222	**2358**	17/04/2016	93270	E	656	NA	no	04/08/2016	T1247	negative	3
223	**2361**	17/05/2016	94700	D	158	female	no	05/08/2016	T1246	negative	3
224	**2363**	17/07/2016	93250	R	77	female	no	05/08/2016	T1267	negative	3
225	**2371**	17/07/2016	94310	D	107	male	no	05/08/2016	T1259	negative	1
226	**2375**	17/07/2016	91550	D	152	NA	no	06/08/2016	T1258	negative	1
227	**2378**	17/07/2016	77200	D	adult	NA	no	06/08/2016	T1257	negative	3
228	**2396**	17/08/2016	75019	E	90	female	no	08/08/2016	T1245	negative	1
229	**2398**	17/08/2016	94500	D	164	NA	no	08/08/2016	T1242	negative	3
230	**2399**	17/10/2016	95100	R	749	male	no	08/08/2016	T1243	negative	4
231	**2400**	17/10/2016	95100	D	NA	NA	no	08/08/2016	T1244	negative	1
232	**2416**	17/10/2016	75020	D	NA	NA	no	10/08/2016	T1254	positive	1
233	**2417**	18/04/2016	94380	D	454	male	no	10/08/2016	T1256	positive	1
234	**2418**	18/05/2016	94400	D	510	male	yes	10/08/2016	T1262	negative	1
235	**2442**	18/05/2016	91330	E	adult	female	no	12/08/2016	T1341	negative	3
236	**2444**	18/06/2016	92350	R	95	NA	no	12/08/2016	T1367	negative	1
237	**2452**	18/07/2016	28500	E	95	NA	no	15/08/2016	T1421	negative	1
238	**2453**	18/07/2016	78150	D	224	male	no	13/08/2016	T1356	negative	3
239	**2454**	18/07/2016	94130	D	NA	NA	no	13/08/2016	T1398	negative	2
240	**2459**	18/09/2016	91380	E	268	NA	no	13/08/2016	T1365	negative	1
241	**2460**	18/09/2016	95480	D	148	NA	no	13/08/2016	T1400	negative	1
242	**2461**	18/09/2016	95480	D	260	NA	no	13/08/2016	T1399	negative	1
243	**2462**	18/10/2016	NA	NA	270	male	no	13/08/2016	T1401	negative	1
244	**2475**	18/10/2016	NA	NA	adult	male	no	15/08/2016	T1402	negative	1
245	**2476**	18/10/2016	NA	D	385	female	no	15/08/2016	T1403	negative	1
246	**2477**	18/10/2016	94290	D	428	female	no	15/08/2016	T1404	negative	1
247	**2487**	18/12/2016	92160	NA	549	female	no	16/08/2016	T1371	negative	1
248	**2492**	18/12/2016	77400	NA	113	female	no	17/08/2016	T1415	positive	1
249	**2496**	19/04/2016	92160	E	124	male	no	17/08/2016	T1418	positive	1
250	**2515**	19/06/2016	94170	E	125	NA	no	19/08/2016	T1416	negative	1
251	**2516**	19/06/2016	92190	D	147	male	yes	19/08/2016	T1417	positive	1
252	**2524**	19/06/2016	92190	E	149	male	no	20/08/2016	T1390	negative	1
253	**2527**	19/06/2016	77220	D	460	male	no	20/08/2016	T1389	negative	1

254	**2534**	19/06/2016	92190	R	550	male	no	21/08/2016	T1351	positive	4
255	**2535**	19/06/2016	77178	D	686	NA	no	21/08/2016	T1340	positive	6
256	**2536**	19/06/2016	60500	D	577	NA	no	21/08/2016	T1350	positive	4
257	**2543**	19/06/2016	95170	R	NA	female	no	21/08/2016	T1387	negative	1
258	**2565**	19/08/2016	91400	D	460	female	no	23/08/2016	T1383	negative	1
259	**2566**	19/08/2016	94000	E	297	female	no	23/08/2016	T1409	negative	1
260	**2567**	20/06/2016	94420	D	347	male	no	23/08/2016	T1384	negative	1
261	**2572**	20/06/2016	60330	D	600	female	no	24/08/2016	T1412	negative	1
262	**2580**	20/07/2016	94500	D	101	male	no	24/08/2016	T1353	negative	2
263	**2581**	20/07/2016	94410	R	403	male	no	24/08/2016	T1352	negative	1
264	**2598**	20/08/2016	78180	E	50	NA	no	26/08/2016	T1411	positive	3
265	**2599**	20/08/2016	91700	D	880	female	no	26/08/2016	T1363	negative	1
266	**2600**	20/09/2016	75016	D	NA	female	no	26/08/2016	T1408	negative	6
267	**2601**	20/11/2016	95180	NA	290	male	no	26/08/2016	T1346	negative	1
268	**2602**	20/11/2016	91540	D	393	female	no	26/08/2016	T1347	negative	1
269	**2638**	20/11/2016	77330		539	female	no	29/08/2016	T1326	positive	1
270	**2639**	21/05/2016	94440	R	1400	female	no	29/08/2016	T1325	positive	4
271	**2642**	21/06/2016	94290	R	101	NA	no	29/08/2016	T1379	positive	4
272	**2647**	21/07/2016	78740	D	85	female	no	31/08/2016	T1377	positive	1
273	**2649**	21/07/2016	78740	E	103	male	yes	31/08/2016	T1378	negative	2
274	**2650**	21/07/2016	77590	D	NA	male	no	30/08/2016	T1424	negative	1
275	**2678**	21/08/2016	95410	NA	151	NA	no	01/09/2016	T1344	negative	2
276	**2688**	21/08/2016	95410	NA	156	NA	no	02/09/2016	T1345	negative	1
277	**2695**	21/08/2016	95410	NA	162	female	no	02/09/2016	T1462	negative	1
278	**2700**	21/08/2016	91200	D	247	NA	no	03/09/2016	T1349	positive	1
279	**2702**	21/09/2016	94160	E	291	female	no	03/09/2016	T1339	positive	1
280	**2703**	21/09/2016	77184	D	294	NA	no	03/09/2016	T1337	positive	5
281	**2704**	21/09/2016	93330	NA	296	male	no	03/09/2016	T1426	positive	4
282	**2705**	21/09/2016	94320	R	582	NA	no	03/09/2016	T1364	negative	3
283	**2711**	21/11/2016	78330	D	222	female	no	03/09/2016	T1405	positive	1
284	**2729**	21/11/2016	60500	D	366	male	no	04/09/2016	T1459	negative	1
285	**2735**	22/04/2016	94550	R	544	female	no	05/09/2016	T1458	positive	1

286	**2736**	22/05/2016	94320	E	409	male	no	05/09/2016	T1457	negative	1
287	**2750**	22/06/2016	94700	D	NA	NA	no	06/09/2016	T1464	negative	1
288	**2752**	22/06/2016	92000	D	NA	NA	no	06/09/2016	T1463	negative	1
289	**2778**	22/10/2016	94190	R	958	male	no	08/09/2016	T1456	negative	3
290	**2781**	22/10/2016	94250	E	adult	female	no	09/09/2016	T1455	negative	1
291	**2783**	22/11/2016	94500	E	214	male	yes	09/09/2016	T1454	negative	1
292	**2799**	23/04/2016	94450	R	689	male	no	10/09/2016	T1453	negative	1
293	**2817**	23/06/2016	78350	D	96	male	no	11/09/2016	T1451	positive	4
294	**2818**	23/06/2016	78350	D	103	male	no	11/09/2016	T1452	positive	1
295	**2824**	23/06/2016	95190	R	133	male	no	12/09/2016	T1448	negative	3
296	**2825**	23/06/2016	91790	E	517	male	no	12/09/2016	T1449	negative	1
297	**2826**	23/06/2016	94430	D	599	male	no	12/09/2016	T1450	negative	2
298	**2839**	23/08/2016	78350	E	91	male	no	14/09/2016	T1489	negative	1
299	**2840**	23/08/2016	78350	E	95	male	no	15/09/2016	T1488	negative	4
300	**2841**	23/08/2016	78350	E	97	female	no	14/09/2016	T1490	negative	1
301	**2874**	23/11/2016	94700	D	443	female	no	15/09/2016	T1502	negative	2
302	**2881**	24/05/2016	94130	R	540	female	no	16/09/2016	T1503	negative	1
303	**2895**	24/06/2016	78860	D	96	male	no	18/09/2016	T1507	negative	1
304	**2896**	24/06/2016	93700	R	99	male	no	18/09/2016	T1501	positive	1
305	**2897**	24/06/2016	95100	R	100	male	no	18/09/2016	T1501b	negative	1
306	**2914**	24/06/2016	93700	R	101	male	no	20/09/2016	T1538	negative	1
307	**2915**	24/06/2016	95100	R	107	female	no	21/09/2016	T1572	negative	1
308	**2919**	24/06/2016	94800	E	107	male	no	21/09/2016	T1571	positive	1
309	**2921**	24/06/2016	93700	R	110	male	no	21/09/2016	T1570	negative	3
310	**2925**	24/06/2016	28700	D	488	male	no	21/09/2016	T1568	negative	1
311	**2961**	24/06/2016	92700	R	524	male	no	25/09/2016	T1579	negative	2
312	**2974**	24/06/2016	95100	R	875	male	yes	26/09/2016	T1638	negative	1
313	**3004**	24/08/2016	77450	D	200	female	no	30/09/2016	T1640	negative	1
314	**3006**	24/08/2016	77450	D	200	female	no	30/09/2016	T1636	negative	1
315	**3019**	24/08/2016	93250	D	623	female	no	02/10/2016	T1637	negative	3
316	**3024**	24/10/2016	77860	R	172	male	no	02/10/2016	T1668	negative	1
317	**3026**	24/10/2016	60500	D	414	male	no	03/10/2016	T1667	positive	5

318	**3032**	25/10/2016	28290	D	358	male	no	04/10/2016	T1669	positive	1
319	**3043**	25/03/2016	77176	R	750	female	yes	05/10/2016	T1681	negative	1
320	**3050**	25/04/2016	94500	R	700	male	yes	07/10/2016	T1682	negative	1
321	**3054**	25/05/2016	92370	R	770	female	no	07/10/2016	T1670	positive	3
322	**3062**	25/06/2016	60240	D	80	male	no	09/10/2016	T1680	positive	1
323	**3063**	25/06/2016	60240	D	90	male	no	09/10/2016	T1666	negative	3
324	**3067**	25/06/2016	60240	R	125	female	no	09/10/2016	T1679	negative	1
325	**3068**	25/06/2016	95500	D	496	NA	no	09/10/2016	T1678	negative	1
326	**3071**	25/07/2016	NA	R	430	female	no	09/10/2016	T1677	negative	1
327	**3080**	25/07/2016	NA	D	430	male	no	10/10/2016	T1743	negative	1
328	**3081**	25/07/2016	78140	R	445	female	no	10/10/2016	T1751	negative	2
329	**3082**	25/07/2016	NA	R	532	male	no	10/10/2016	T1741	negative	2
330	**3083**	25/07/2016	93340	R	694	male	no	10/10/2016	T1675	negative	3
331	**3090**	25/08/2016	91600	NA	76	female	no	11/10/2016	T1676	negative	1
332	**3092**	25/09/2016	95230	NA	163	female	no	11/10/2016	T1760	negative	1
333	**3095**	26/04/2016	94100	D	579	female	yes	12/10/2016	T1744	negative	1
334	**3097**	26/06/2016	78700	R	85	female	no	12/10/2016	T1771	negative	1
335	**3100**	26/06/2016	92250	R	102	female	no	12/10/2016	T1742	positive	3
336	**3101**	26/06/2016	91090	R	109	female	no	12/10/2016	T1770	negative	1
337	**3103**	26/06/2016	NA	D	110	male	no	13/10/2016	T1740	negative	3
338	**3107**	26/06/2016	95150	R	113	male	no	13/10/2016	T1745	negative	2
339	**3109**	26/06/2016	NA	D	159	NA	no	13/10/2016	T1746	negative	1
340	**3116**	26/06/2016	94520	R	759	female	no	14/10/2016	T1755	negative	1
341	**3129**	26/06/2016	77420	E	114	male	no	16/10/2016	T!747	negative	1
342	**3133**	26/06/2016	78700	D	NA	female	no	16/10/2016	T1739	negative	1
343	**3135**	26/06/2016	93000	E	546	NA	no	16/10/2016	T1748	negative	1
344	**3136**	26/07/2016	78500	D	65	male	no	16/10/2016	T1749	negative	1
345	**3144**	26/07/2016	78500	D	70	male	no	17/10/2016	T1750	negative	2
346	**3147**	26/07/2016	92330	D	NA	NA	no	17/10/2016	T1761	negative	1
347	**3150**	26/08/2016	91600	D	66	female	no	17/10/2016	T1759	negative	1
348	**3155**	26/08/2016	91600	NA	80	male	no	18/10/2016	T1758	negative	1
349	**3156**	26/08/2016	94140	D	790	male	no	18/10/2016	T1738	negative	1

350	**3157**	26/08/2016	94140	E	840	male	no	18/10/2016	T1737	negative	3
351	**3158**	26/09/2016	92380	E	111	NA	no	18/10/2016	T1736	negative	3
352	**3182**	27/06/2016	95740	D	40	male	no	22/10/2016	T1883	negative	1
353	**3185**	27/06/2016	77220	D	150	female	no	22/10/2016	T1839	negative	1
354	**3189**	27/06/2016	77220	D	165	female	no	24/10/2016	T1841	negative	1
355	**3193**	27/06/2016	77220	D	170	female	no	24/10/2016	T1840	negative	2
356	**3212**	27/06/2016	77220	R	191	female	no	27/10/2016	T1837	negative	1
357	**3213**	27/06/2016	27700	E	575	female	no	27/10/2016	T1834	negative	3
358	**3214**	27/06/2016	94160	E	602	NA	no	27/10/2016	T1838	positive	2
359	**3216**	27/07/2016	92190	R	312	female	no	27/10/2016	T1884	negative	1
360	**3222**	27/07/2016	NA	D	650	female	no	28/10/2016	T1835	negative	1
361	**3223**	27/07/2016	78510	D	960	male	no	28/10/2016	T1836	negative	2
362	**3224**	27/07/2016	92370	D	adult	NA	no	28/10/2016	T1882	negative	1
363	**3228**	27/07/2016	78320	D	260	NA	no	29/10/2016	T1833	negative	1
364	**3253**	27/10/2016	95270	D	210	female	no	02/11/2016	T1865	negative	1
365	**3258**	27/10/2016	95270	NA	264	male	no	28/10/2016	T1877	negative	1
366	**3267**	27/10/2016	77000	E	323	female	no	04/11/2016	T1867	negative	2
367	**3268**	27/10/2016	95270	NA	340	female	no	04/11/2016	T1868	negative	2
368	**3269**	28/04/2016	95170	E	589	male	no	04/11/2016	T1873	negative	1
369	**3271**	28/05/2016	91180	E	adult	female	no	04/11/2016	T1866	negative	1
370	**3283**	28/07/2016	77000	D	208	female	no	06/11/2016	T1930	negative	2
371	**3284**	28/10/2016	93200	D	188	NA	no	06/11/2016	T1942	negative	1
372	**3287**	28/10/2016	94100	D	197	female	no	07/11/2016	T1929	positive	1
373	**3290**	28/10/2016	94100	NA	221	male	no	08/11/2017	T1941	negative	1
374	**3304**	28/11/2016	60230	D	147	female	no	11/11/2016	T1963	negative	1
375	**3306**	28/11/2016	93290	NA	559	male	no	12/11/2016	T1965	negative	1
376	**3310**	28/11/2016	94420	D	Juvenile	male	no	12/11/2016	T1964	negative	2
377	**3317**	29/03/2016	NA	R	503	female	no	13/11/2016	T1962	negative	1
378	**3326**	29/04/2016	92600	D	428	female	no	15/11/2016	T1966	negative	1
379	**3328**	29/04/2016	77122	D	544	male	yes	16/11/2016	T1969	positive	5
380	**3330**	29/06/2016	95520	E	93	male	no	16/11/2016	T1968	positive	1
381	**3332**	29/06/2016	NA	R	542	male	no	16/11/2016	T1961	positive	1

382	**3347**	29/06/2016	95800	D	580	male	yes	20/11/2016	T1999	positive	2
383	**3348**	29/06/2016	NA	R	592	female	no	20/11/2016	T1998	positive	3
384	**3352**	29/08/2016	91600	NA	63	male	no	20/11/2016	T1997	positive	1
385	**3353**	29/08/2016	95210	D	95	female	yes	21/11/2016	T2008	positive	2
386	**3357**	29/08/2016	95210		138	male	yes	21/11/2016	T2009	negative	1
387	**3359**	29/10/2016	91420	D	632	male	no	22/11/2016	T2153	positive	1
388	**3361**	29/11/2016	78570	D	305	female	no	23/11/2016	T2152	negative	1
389	**3380**	30/03/2016	95420	R	974	male	no	28/11/2016	T2099	negative	1
390	**3381**	30/06/2016	NA	D	37	NA	no	28/11/2016	T2151	negative	1
391	**3385**	30/06/2016	NA	D	47	NA	no	28/11/2016	T2098	negative	1
392	**3391**	30/06/2016	NA	D	790	NA	no	29/11/2016	T2097	negative	1
393	**3397**	30/06/2016	NA	D	49	NA	no	30/11/2016	T2096	positive	3
394	**3398**	30/06/2016	72340	D	63	NA	no	30 11 2016	T2094	positive	3
395	**3399**	30/06/2016	91140	R	1095	male	no	30/11/2016	T2093	negative	1
396	**3404**	30/07/2016	60660	R	92	NA	no	01/12/2016	T2092	negative	1
397	**3408**	30/07/2016	60660	R	94	male	no	02/12/2016	T2150	negative	1
398	**3409**	30/07/2016	60660	NA	97	female	no	03/12/2016	T2091	negative	1
399	**3414**	30/07/2016	60660	R	98	female	no	03/12/2016	T2090	negative	1
400	**3415**	30/07/2016	94700	D	328	NA	no	04/12/2016	T2089	positive	1
401	**3419**	30/08/2016	95320	D	45	male	no	06/12/2016	T2095	negative	1
402	**3420**	30/08/2016	95320	R	48	female	no	06/12/2016	T2154	negative	1
403	**3421**	30/08/2016	94130	R	380	NA	no	06/12/2016	T2088	negative	1
404	**3435**	30/09/2016	94200	D	119	NA	no	09/12/2016	T2148	negative	1
405	**3436**	30/09/2016	95140	E	216	NA	no	09/12/2016	T2149	negative	1
406	**3437**	30/11/2016	95390	NA	229	male	no	09/12/2016	T2147	negative	1
407	**3440**	30/11/2016	95390	NA	288	female	yes	10/12/2016	T2146	positive	1
408	**3442**	30/11/2016	95110	R	NA	male	yes	10/12/2016	T2145	negative	1
409	**3444**	31/05/2016	78280	D	464	male	no	11/12/2016	T2144	negative	1
410	**3452**	31/07/2016	91700	R	650	male	no	15/12/2016	T2188	negative	1
411	**3458**	31/07/2016	94000	E	895	male	no	18/12/2016	T2189	negative	1
412	**3460**	31/07/2016	94700	R	1100	female	yes	18/12/2016	T2190	positive	2

PAPER 4

Population biology of *Trichophyton erinacei*

Čmoková A, Kolařík M, Dobiáš R, Cabañes FJ, Guillot J, Kano R, Kuklová I, Lysková P, Nenoff P, Mencl K, Mallátová N, Risco-Castillo V, Stubbe D, Uhrlaß S, & Hubka V.

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Host-driven subspeciation in the hedgehog fungus, *Trichophyton erinacei*, an emerging cause of human dermatophytosis

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ABSTRACT

Trichophyton erinacei is a main cause of dermatophytosis in hedgehogs and is increasingly reported from human infections worldwide. This pathogen was originally described in the European hedgehog (*Erinaceus europaeus*) but is also frequently found in the African four-toed hedgehog (*Atelerix albiventris*), a popular pet animal worldwide. Little is known about the taxonomy and population genetics of this pathogen despite its increasing importance in clinical practice. Notably, whether there are different populations or even cryptic species associated with different hosts or geographic regions is not known. To answer these questions, we collected 161 isolates, performed phylogenetic and population-genetic analyses, determined mating-type, and characterised morphology and physiology. Multigene phylogeny and microsatellite analysis supported *T. erinacei* as a monophyletic species, in

contrast to highly incongruent single-gene phylogenies. Two main subpopulations, one specific mainly to *Atelerix* and second to *Erinaceus* hosts, were identified inside *T. erinacei*, and slight differences in the size of microconidia and antifungal susceptibilities were observed among them. Although the process of speciation into two lineages is ongoing in *T. erinacei*, there is still gene flow between these populations. Thus, we present *T. erinacei* as a single species, with notable intraspecies variability in genotype and phenotype. The data from wild hedgehogs indicated that sexual reproduction in *T. erinacei* and de novo infection of hedgehogs from soil are probably rare events and that clonal horizontal spread strongly dominates. The molecular typing approach used in this study represents a suitable tool for further epidemiological surveillance of this emerging pathogen in both animals and humans. The results of this study also highlighted the need to use a multigene phylogeny ideally in combination with other independent molecular markers to understand the species boundaries of dermatophytes.

KEYWORDS: Epizootic fungal infections, Microsatellite typing, Multigene phylogeny, Population genetics, Skin infections, *Trichophyton benhamiae* complex, Zoophilic dermatophytes

INTRODUCTION

Trichophyton erinacei and other species from the *Trichophyton benhamiae* complex are mainly zoophilic fungal pathogens that are frequently transmitted to humans. These zoonotic infections, usually manifesting as highly inflammatory tinea corporis or tinea capitis, are common in pet owners and contribute significantly to numerous occupational infections in farmers, workers in livestock production, laboratory workers, pet shop workers, and other professions that require contact with animals (Halsby et al. 2014, Hubka et al. 2018b). In the last decade, pathogens from the *T. benhamiae* complex have attracted considerable attention mainly due to the epizootic / epidemic spread of dermatophytosis caused by *T. benhamiae* var. *luteum* in guinea pigs and humans in Europe (Berlin et al. 2020, Čmoková et al. 2020). Similarly, the hedgehog pathogen *T. erinacei* is increasingly reported as a cause of dermatophytosis in hedgehog breeders or owners, but with a lower incidence. Unlike *T. benhamiae* var. *luteum*, the growing incidence of *T. erinacei* is not due to the emergence of a new virulent pathogen but rather to changes in preferences breeders have who increasingly buy hedgehogs as pets (Fig. 1). A worldwide distribution of *T. erinacei* is documented by numerous tinea cases from Europe (English et al. 1962, Romano et al. 2001, Schauder et al. 2007, Weishaupt et al. 2014, Perrier & Monod 2015, Abarca et al. 2017, Kargl et al. 2018, Lysková et al. 2018, Rivaya et al. 2020), Asia (Mochizuki et al. 2005, Lim et al. 2009, Rhee et al. 2009, Hsieh et al. 2010, Hui et al. 2017, Choi et al. 2018, Kim et al. 2018, Al Masaoodi et al. 2020, Ogawa et al. 2020, Watabe et al. 2021), America (Rosen 2000, Concha et al. 2012, Eid et al. 2014, Alejandra et al. 2018, Frantz et al. 2020, Rodríguez-Silva et al. 2021, Walsh et al. 2021) and Africa (Drira et al. 2015). Most cases described in literature were transmitted from pet African pygmy hedgehogs (syn. four-toed hedgehog; *Atelerix albiventris*). However, *T. erinacei* is also reported in wild European hedgehogs, i.e., West European hedgehogs (*Erinaceus europaeus*) and

Northern white-breasted hedgehogs (*Erinaceus roumanicus*), which may represent risks for people, especially in wildlife rehabilitation centres or for dogs that may occasionally encounter hedgehogs in places of their hibernation (Le Barzic et al. 2021). Infections due to *T. erinacei* most often manifest as tinea manuum or tinea corporis localized on fingers, hands, wrists, or forearms (Fig. 2). Tinea faciei, kerion type of tinea capitis, tinea barbae and onychomycosis have also been documented in the abovementioned case reports.

Similar to *T. benhamiae* var. *luteum* in guinea pigs (Berlin et al. 2020), available epidemiological surveys showed high infection rates of *T. erinacei* in hedgehogs. The prevalence in *E. europaeus* ranged between 20–30 % in wild hedgehogs in France, Poland and Great Britain (English & Morris 1969, Gnat et al. 2021) and up to 45 % in New Zealand (Smith & Marples 1964). The prevalence in *A. albiventris* ranged between 22–38 % in wild animals in Africa (Gregory & English 1975, Gregory et al. 1978) and 39–50 % in pet hedgehogs in Japan and Spain (Takahashi et al. 2003, Abarca et al. 2017). Hedgehogs are also the source of other potentially zoonotic bacterial, viral and fungal pathogens. Among these, *Staphylococcus aureus*, and especially methicillin-resistant *S. aureus* (MRSA) strains, belong to the most feared pathogens which have a high prevalence in hedgehogs (Bengtsson et al. 2017, Rasmussen et al. 2019). Interestingly, it was demonstrated that dermatophyte-infected hedgehogs provide a natural selective environment for MRSA because *T. erinacei* produces two antibiotics from the penicillin class (Dube et al. 2021, Larsen et al. 2022). Specific lineages of MRSA derived from hedgehogs have existed even before the era of antibiotics and their spread between livestock and humans has been confirmed (Larsen et al. 2022).

The geographic distribution of *T. erinacei* follows the distribution of hedgehogs (*Erinaceidae*) in Afro-Eurasia, with the highest diversity in Asia, where four of five genera occur, while the fifth genus *Atelerix* is found exclusively in Africa. Only two species of the genus *Erinaceus* are widespread in Europe, i.e., *E. europaeus* occurring in Western Europe and *E. roumanicus* in Eastern Europe (He et al. 2012), with the contact zone in Central Europe (Curto et al. 2019). Imperceptible morphological differences between these two species, especially in the contact zone, make exact identification difficult for non-experts (Černá Bolfíková et al. 2020). In addition to natural habitats, hedgehogs have been introduced in many non-native areas.

The genus *Erinaceus* has been introduced to New Zealand, while *A. albiventris* and *Hemiechinus auratus* are considered invasive species in Spain. Worldwide, *A. albiventris*, *A. algirus* and their hybrids are kept as pets (Reeve 1994, He et al. 2012) or as research animals in laboratories (Lawn et al. 1995).

Erinaceus and *Atelerix* hedgehogs have been reported to be carriers of *T. erinacei* isolates with different genotypes and mating type gene idiomorphs, respectively (Takashio 1979, Takahashi et al. 2003, Schauder et al. 2007, Heidemann et al. 2010), suggesting that partially isolated populations co-evolved with different hosts. However, whether the level of differentiation of these populations could meet the criteria for delimitation of separate species is not known. To answer this question, we col- lected *T.*

erinacei isolates from different hedgehog genera and cases of human infections. The population structure and characteristics (e.g., genotype diversity and random mating) were examined using multilocus microsatellite typing (MLMT) and multilocus sequence typing (MLST) approaches. The association between population structure, host, geographic origin, antifungal susceptibility, mating-type gene distribution and phenotype was investigated to reveal patterns suggestive of biological divergence or (sub)speciation.

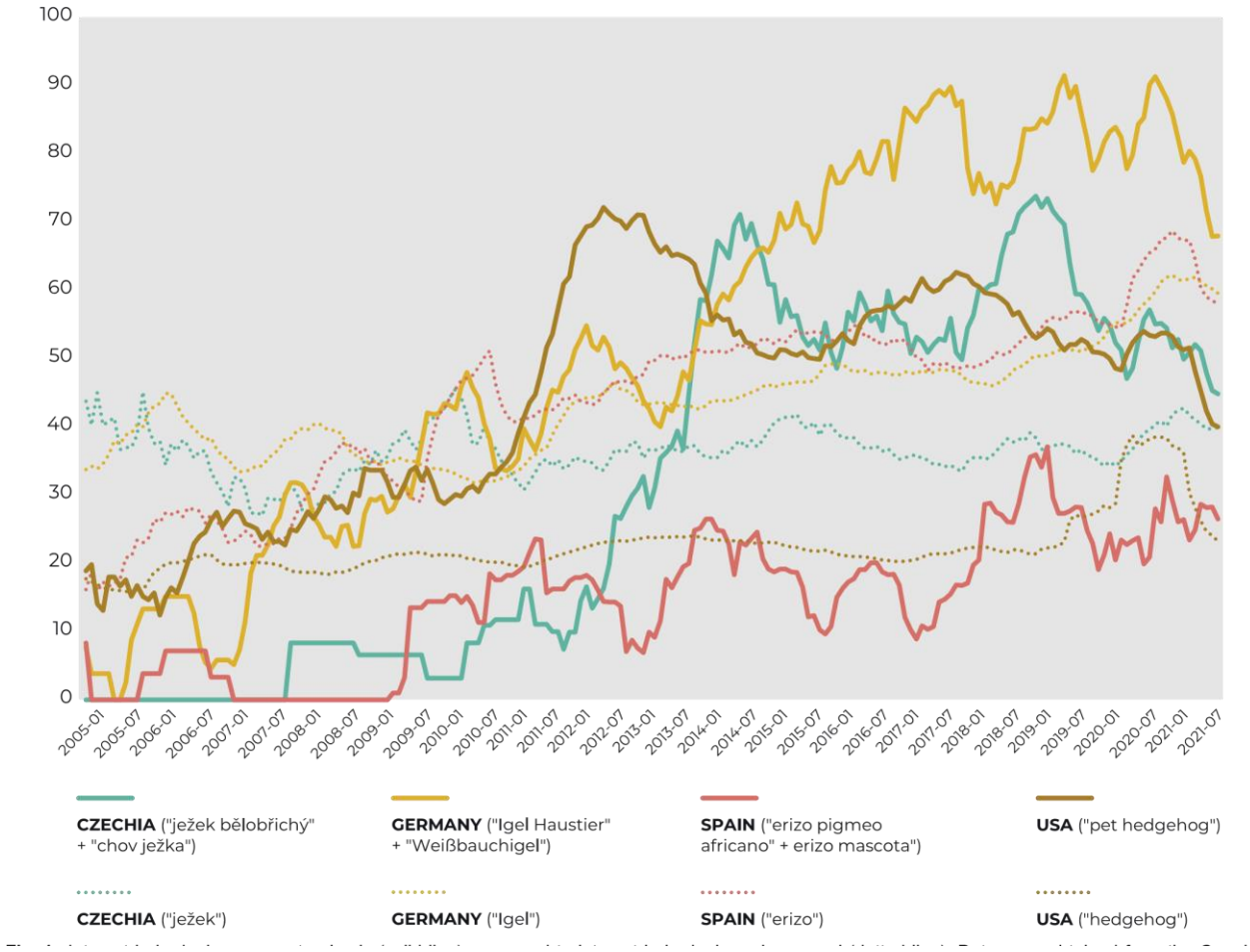

Fig. 1 Interest in hedgehogs as a pet animals (solid line) compared to interest in hedgehogs in general (dotted line). Data were obtained from the Google Trends and show relative interest scaled on a range of 0 to 100 during years 2005–2021 (measured as number of searches during half-year). Data are shown for four countries and key words are listed in parentheses.

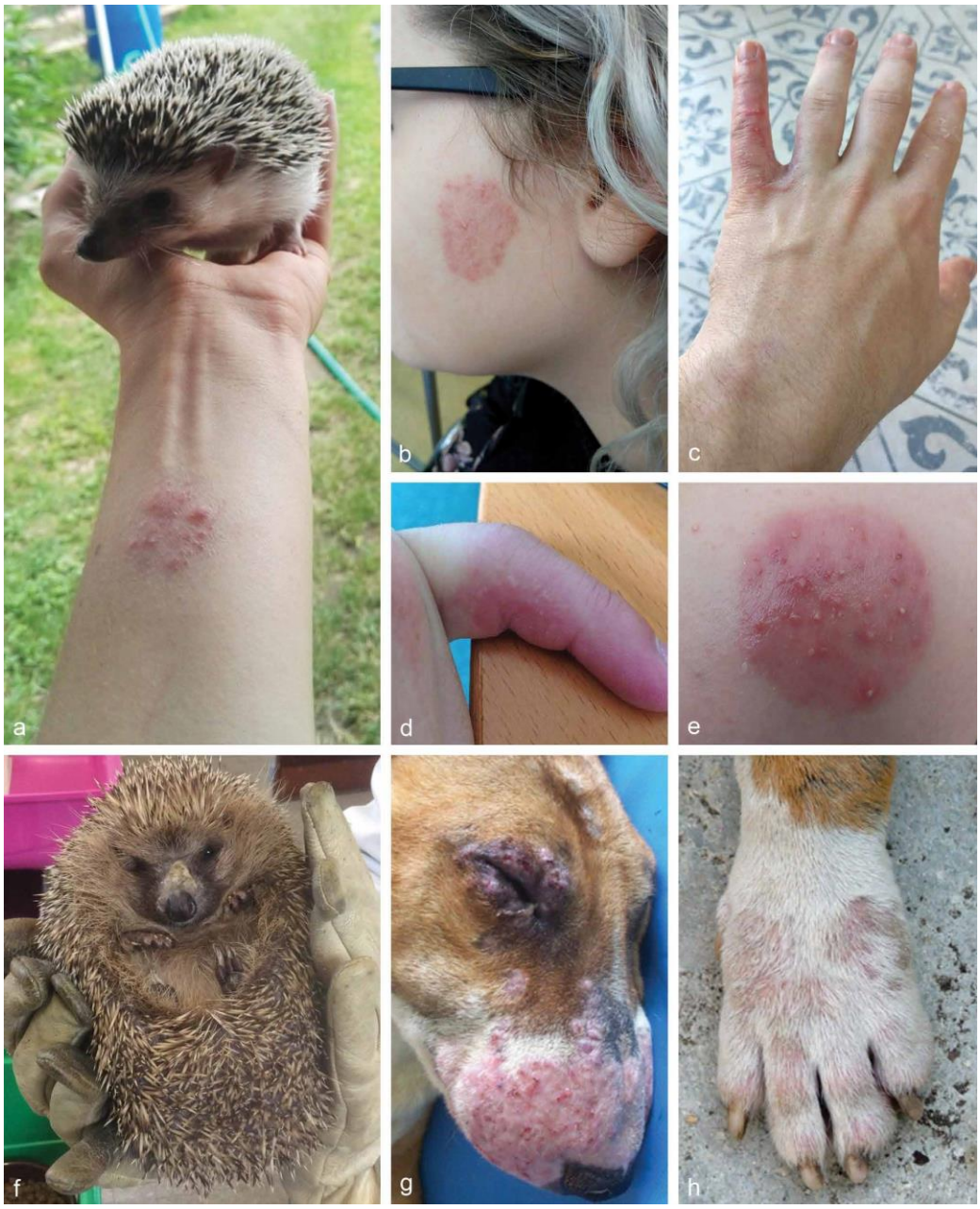

Fig. 2 Clinical manifestation of human (A-E) and animal (F-H) infections caused by *Trichophyton erinacei*. Tinea corporis on the left forearm (26-year old woman), erythematous lesion covered with pustules, contact with a pet hedgehog (*Atelerix albiventris*) shown on the same picture, isolate IDE 740/19 (A); tinea faciei (20-year-old woman), erythematous, scaly, sharply demarcated lesion on the left cheek, contact with a pet hedgehog (*A. albiventris*), isolate CCF 5775 (B); erythematous, scaly lesion on the little finger (20-year-old man), contact with a pet hedgehog (*A. albiventris*), isolate SK 1826/19 (C, D); tinea corporis (24-year-old woman) on the outer side of the right thigh, lesion approximately 5 cm in diameter, pustules in the center, contact with pet hedgehog (*A. albiventris*), isolate CCF 5209 (E); hedgehog (*Erinaceus europaeus*) with erythematous dermatitis and scaly lesions above the muzzle (F); alopecia, erythema and pustules affecting head and limbs of a 4-year-old Bull terrier, isolate CBS 124411 (G, H).

MATERIALS AND METHODS

Source of isolates

The isolates (n = 161) were collected from humans and hedgehogs and obtained in collaboration with clinical laboratories, hospitals, universities and culture collections (Table S1): National Veterinary School of Alfort (France); Autonomous University of Barcelona (Spain), Laboratory for Medical Microbiology (Germany); College of Veterinary Medicine, College of Bioresource Sciences, Nihon University (Japan); University of Turin (Italy); BCCM/IHEM Fungi Collection: Human and Animal Health (Belgium); CBS culture collection housed at the Westerdijk Institute (The Netherlands); and various institutions in the Czech Republic, i.e., Institute of Public Health in Ostrava and Ústí nad Labem, Hospital České Budějovice, General University Hospital in Prague, University Hospital in Pilsen, and the University Hospital Olomouc.

Selected isolates were deposited into the Culture Collection of Fungi (CCF), Department of Botany, Charles University, Prague, Czech Republic; and German Collection of Microorganisms and Cell Cultures (DSMZ), Leibniz Institute, Braunschweig, Germany.

Molecular studies

DNA was extracted from 7-d-old colonies using the QuickDNA™ Fungal/Bacterial Miniprep kit (Zymo Research, USA). Amplification of the ITS rDNA region (ITS1-5.8S-ITS2 cluster) was performed using the primer set ITS1F and ITS4 (White et al. 1990, Gardes & Bruns 1993), the partial *gapdh* gene encoding glyceraldehyde-3-phosphate dehydrogenase using primers GPDF and GPDR (Kawasaki et al. 2011), the partial *tubb* gene encoding β-tubulin using primers Bt2a and Bt2b (Glass & Donald son 1995), and the partial *tef1-α* gene encoding translation elongation factor 1-α using primers EF-DermF and EF-DermR (Mirhendi et al. 2015). Polymerase chain reaction (PCR) conditions were described by Sklenář et al. (2021). PCR products were purified using a Sap-Exo Kit (Jena Bioscience, Germany) and subsequent automated sequencing was performed at BIOCEV (Vestec, Czech Republic) using both terminal primers. The DNA sequences obtained in this study (unique genotypes only) were deposited into the GenBank database (www.ncbi.nlm.nih.gov) under the accession numbers listed in Table 1.

Table 1 Sequence accession numbers to unique multilocus genotypes of *Trichophyton erinacei*.

MLST genotype	Representative strain	Accession numbers				Corresponding MLMT genotype(s)
		ITS (genotype)	*tef1-α* (genotype)	*gapdh* (genotype)	*tubb* (genotype)	
Gl (35 strains)	CCF 5209	MZ314454 (a)	MZ320337 (a)	MZ320332 (a)	MZ320327 (a)	Ml -M3
G2 (I strain)	CBS 124411	MZ314453 (b)	MZ320336 (a)	MZ320331 (a)	MZ320326 (a)	M5-M6
G3 (4 strains)	DSM 104923	MZ314456 (c)	MZ320339 (a)	MZ320334 (a)	MZ320329 (a)	M7-M9
G4 (8 strains)	CBS 511.73^{T}	LR794136 (a)	LR794267 (b)	LR794242 (a)	LR794292 (a)	MIO
G5 (23 strains)	CCF 6427	MZ314455 (a)	MZ320338 (a)	MZ320333 (b)	MZ320328 (a)	Ml-ST

MLST, multilocus sequence typing; MLMT, multilocus microsatelitte typing

Table 2 Characterisation of alignments, partition-merging results and best substitution model for each partition according to Bayesian information criterion

Alignment	Length (bp)	Variable position	Parsimony informative Sites	Phylogenetic method	Partitioning scheme (substitution model)
ITS, *gapdh*, *tubb* & *tefl-α* (tree: Fig. 3)	2278	243	154	Maximum likelihood (ML)	Six partitions: ITS1 & ITS2 (TrN+G); 5.8S (K80); 1st codon positions of *gapdh* & *tef1-α* (F81 +I); 1st codon positions of *tubb* & 2nd codon positions of *gapdh*, *tubb* & *tef1-α* (JC); 3rd codon positions of *gapdh*, *tubb* & *tef1-α* (HKY+I); introns of *gapdh*, *tubb* & *tef1-α* (K80+I+G)
ITS, *gapdh*, *tubb* & *tefl-α* (tree: Fig. 3)	2278	243	154	Bayesian inference	Five partitions: ITS1 & ITS2 & 3rd codon positions of *gapdh*, *tubb* & *tef1-α* (HKY+G); 5.8S (K80); 1st codon positions of *gapdh* & *tef1-α* (F81 +I); 1st codon positions of *tubb* & 2nd codon positions of *gapdh*, *tubb* & *tef1-α* (JC); introns of *gapdh*, *tubb* & *tef1-α* (K80+I+G)
ITS (tree: Fig. 8)	592	88	53	ML	Two partitions: ITS1 & ITS2 (TrN+G); 5.8S (K80)
gapdh (tree: Fig. 8)	563	54	31	ML	Three partitions: 1st codon positions (F81+l); 2nd codon positions (JC); 3rd codon positions & introns (HKY+G)
tubb (tree: Fig. 8)	463	41	24	ML	Three partitions: 1st & 2nd codon positions (JC); 3rd codon positions (HKY+I); introns (K80)
tefl-α (tree: Fig. 8)	660	60	46	ML	Three partitions: 1st & 2nd codon positions (JC); 3rd codon positions (HKY); intronsv(K80+G)

A fragment of the *MAT1-1-1* gene encoding a protein with an alpha-domain motif was amplified with the primer pairs MF3 and MF6 or MF3 and MF4, and a fragment of the *MAT1-2-1* gene encoding the high mobility group (HMG) domain was amplified with the primers TmHMG3S and TmHMG3R (Kano et al. 2012, Symoens et al. 2013). Several PCR products of each MAT idiomorph were subjected to DNA sequencing for verification of specificity, and the remaining samples were visualized on an electrophoretogram (1 % agarose gel with 0.5 µg/ mL ethidium bromide) and identified according to the specific lengths of PCR products.

Subtyping of *T. erinacei* using microsatellite markers was per- formed using the previously developed typing scheme for species from the *T. benhamiae* clade (Čmoková et al. 2020). The reaction volume of 5 µL for PCR contained 50 ng of DNA, 0.5 µL of the mixture of primers and 2.5 µL of Multiplex PCR Master Mix (Qiagen, Germany). The PCR conditions were chosen according to the manufacturer's recommendations. The PCR products (diluted in water 1 : 25) were mixed with 10 µL of deionized formamide and 0.2 µL of the GeneScan™ 600 LIZ size standard, denatured for 5 min at 95 °C, and subsequently analysed using an ABI 3100 Avant Genetic Analyzer.

Phylogenetic analysis

Alignments of the ITS, *gapdh*, *tubb* and *tef1-α* regions were performed using the FFT-NS-i option implemented in the MAFFT online service (Katoh et al. 2017). The alignments were trimmed, concatenated and then analysed using maximum likelihood (ML) and Bayesian inference (BI) methods. Suitable partitioning schemes and substitution models (Bayesian information criterion) for the analyses were selected using a greedy strategy implemented in PartitionFinder v. 2 (Lanfear et al. 2017) with settings allowing introns, exons, codon positions and segments of the ITS region to be independent datasets. The optimal partitioning schemes for each analysed dataset along with basic alignment characteristics are listed in Table 2.

ML trees were constructed with IQ-TREE v. 1.4.4 (Nguyen et al. 2015) with nodal support determined by ultrafast bootstrapping (BS) with 100 000 replicates. Trees were rooted with *Trichophyton rubrum*. Bayesian posterior probabilities (PP) were calculated for the concatenated dataset using MrBayes v. 3.2.6 (Ronquist et al. 2012). The analysis ran for 10^7 generations, two parallel runs with four chains each were used, every 1000th tree was retained, and the first 25 % of trees were discarded as burn-in. The convergence of the runs and effective sample sizes were checked in Tracer v. 1.6 (http://tree.bio.ed.ac.uk/software/tracer).

Statistical analysis of microsatellite data

Allele and binary data matrices were created using GeneMarker v 1.51 (SoftGenetics, LLC, State College, PA, USA). The allele data matrix was reduced to ≤ 5 samples from each sampling site to minimize the effect of uneven sampling and analysed using a Bayesian model-based clustering algorithm with a clustering number (K) ranging from 1 to 10 in the software STRUCTURE (Pritchard et al. 2000). The admixture model and burn-in of 100 000 iterations followed by 1 M additional Markov chain Monte Carlo iterations were used to calculate 10 simulation runs. The optimal clustering number (K) was estimated by calculating ΔK (Evanno et al. 2005) using the script structure-sum (Ehrich 2006) in R v. 3.3.4 (R Core Team 2016).

To estimate the similarities between individuals, Jaccard's similarity coefficient was calculated on the previously generated binary matrix using the program FAMD (Schlueter & Harris 2006). A neighbour-joining tree based on Jaccard's similarity coefficient matrix was constructed and genetic distances were calculated using the same software. Genetic distances were used for the construction of the NeighborNet network in SplitsTree v. 4 (Huson & Bryant 2006).

The degree of gene flow among clusters was estimated for seven loci on the clone-corrected dataset using a pairwise fixation index (F_{ST}) calculated in Arlequin (Schneider et al. 2000). The degree of clonality or recombination within populations was estimated by calculating the index of association (I_A) in MultiLocus v. 1.3 (Agapow & Burt 2001) using a binary matrix (uncorrected and clone corrected dataset) and a matrix based on both microsatellite and sequence data. The index of association I_A is

estimated to be nearly zero or zero if no linkage is noticed between the alleles of different loci (randomly distributed alleles); in that case random mating (panmixis) is suggested. The values observed in the examined populations were compared to those artificially generated by the 10 000 randomisations. The null hypothesis of recombination and linkage equilibrium is supported when observed and simulated values are close ($p > 0.05$), while significant differences between values support linkage disequilibrium and clonality.

Genetic diversity within population(s) was calculated from the frequencies of genetically distinct individuals using Nei's genotype diversity (Dg), and from the frequencies of alleles at indivi- dual loci using Nei's gene diversity (D) (Nei 1987, Kosman 2003). To obtain diversity values comparable between the clusters, the effective number of genotypes (G_{eff}) (Parker Jr 1979) was calculated based on the number of equally abundant genotypes necessary to reflect the value of a diversity measure. All three population indices (Dg, D, G_{eff}) were calculated from the binary data matrix using script AFLPdat (Ehrich 2006) in R v. 3.0.2.

Phenotypic studies

At least two randomly selected isolates from each MLST genotype (if available) were subjected to a more detailed analysis of macromorphology on Sabouraud glucose agar (SGA, Oxoid, Basingstoke, UK), malt extract agar (MEA, Oxoid, Basingstoke, UK) and potato dextrose agar (PDA, Himedia, Mumbai, India) at 25 °C. In addition, the growth on SGA at 30 and 37 °C was measured. The micromorphological features were measured in the same isolates and were recorded from cultures growing on MEA after 14–21 d of cultivation. The dimensions of conidia were recorded at least 25 times for each isolate.

The macromorphology of the colonies was documented using an Olympus SZ61, and colony colours were determined using the hexadecimal colour codes assigned according to the website https://coolors.co. Micromorphology was documented using an Olympus BX-51 microscope. Statistical differences in the size of spores were tested with one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test in R v. 3.3.4 (R Core Team 2016).

Statistical analysis of the antifungal susceptibility data

The raw data published by Shamsizadeh et al. (2021) were used for statistical analysis. The isolates were divided into two groups according to their assignment to the two clusters based on STRUCTURE analysis. Statistical differences in antifungal susceptibilities to eight antifungals (amorolfine, ciclopirox, efinaconazole, itraconazole, luliconazole, griseofulvin, terbinafine and tavaborole) were tested with one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test (Tukey's HSD) in R v. 3.3.4 (R Core Team 2016).

RESULTS

Genotyping

In total, 161 isolates of *T. erinacei* were subjected to multilocus microsatellite typing (MLMT). Seven out of 10 markers, developed by Čmoková et al. (2020), were successfully amplified in *T. erinacei* (Table S1). A total of 71 samples were selected for multilocus sequence typing (MLST) using four genes (ITS, *gapdh*, *tubb* and *tef1-α*). The reduction in number was at the expense of samples from France, where we observed a high degree clonality among samples using MLMT.

Using the MLST approach, we observed a single genotype in *tubb*, two in *gapdh* and *tef1-a*, and three in ITS, which resulted in five multilocus genotypes designated G1–G5 (Fig. 3). Microsatellite markers showed higher levels of polymorphism 10 genotypes, M1-M10) in comparison to MLST. The most potent microsatellite locus was CT21b (5 genotypes), followed by TAG16 (4 genotypes), TCA16, TC17A, TC20 (3 genotypes each) and CT21 (1 genotype, monomorphic). The plot in Fig. 4 shows the dependence of genotype diversity as a function of an increasing number of microsatellite markers. From the course of the curve, enough loci (n = 7) were used to uncover the genetic diversity present in the examined set of *T. erinacei* strains.

Bayesian approach-based analysis supported the distribution of *T. erinacei* isolates (n = 161) into two clusters, designated here as cluster 1 and cluster 2, according to the ΔK peak (Fig. 5). Cluster 1 consists of two MLMT genotypes (M1 and M2) that correspond to MLST genotype G1 (except for IHEM 25164, which belongs to cluster 2). This cluster contains isolates exclusively (n = 33) from African hedgehogs and their owners (Fig. 3). M1 genotype isolates exhibited only the *MAT1-2-1* idiomorph and originated from Europe, while two M2 genotype isolates exhibited the *MAT1-1-1* idiomorph and originated from Japan. The induction of a sexual state by crossing opposite mating-type strains of M1 and M2 genotypes was not successful on agar medium supplemented with *Guizotia abyssinica* seeds at 20 °C (Symoens et al. 2013, Čmoková 2015). Strain CBS 124411 showed a high level of admixture between clusters 1 and 2 (Fig. 5) but was assigned to cluster 2 by STRUCTURE. This strain is the only representative MLMT genotype M4 (MLST genotype G2) and was isolated from a dog dermatophytosis (Fig. 2) in Italy, and transmission from wild hedgehog of the genus *Erinaceus* is presumed.

Similar grouping of microsatellite genotypes was observed when the NeighborNet network was constructed with FAMD software and visualized in SplitsTree 4 using the Jaccard index-based distance matrix (Fig. 6). Genotypes M1 and M2 clustered together, and the next closest genotype was M4, a transitional genotype between cluster 1 and 2. More diversified cluster 2 consisted of eight MLMT genotypes M3–M10 corresponding to strains from the remaining MLST genotypes G2–G5 (Fig. 3, 6). The cluster comprised mostly strains originating from hedgehogs of the genus *Erinaceus* in Europe, except for two isolates from wild hedgehogs of the genus *Atelerix* in Kenya (IHEM 25146) and Ivory Coast (IHEM 25164).

Three German and one French isolate belonged to the M5 and M6 genotypes, respectively, and MLST genotype G3, all isolated from *Erinaceus* hedgehogs or human infection contracted from them.

The old strains isolated before 2000, including the ex-type strain of *T. erinacei* CBS 511.73 and the ex-type strain of *T. pro liferans* CBS 474.76, mostly belonged to MLST genotype G4 and microsatellite genotypes M7–M9 (Fig. 3, 6). These strains were iso- lated from hedgehogs of the genera *Erinaceus* and *Atelerix* (strain from Kenya, IHEM 25146) and included strains of both mating types (mating experiments were not successful). The majority of strains from cluster 2 were. segregated to the M10 genotype corresponding to MLST genotype G5. These strains originated exclusively from *Erinaceus* hedgehogs from France, and all showed *MAT1-1-1* gene idiomorph

Population structure

Both clusters were genetically uniform as evidenced by low values of Nei's gene diversity (D), especially in cluster 1 (D = 0.07) compared to cluster 2 (D = 0.22). The low Nei's genotype diversity index in cluster 1 (Dg = 0.01) and cluster 2 (Dg = 0.22) reflects that they consisted of several abundant clones that were overrepresented due to uneven sampling. Clonal reproduction prevails in both clusters, which is supported by a low effective number of genotypes (Geff = 1.1 and 1.3, respectively) in comparison with the observed number of genotypes (G = 2 and 8, respectively).

The first cluster of *T. erinacei* consists of only two microsatellite genotypes, which is not enough to determine linkage among markers and make conclusions about the presence of sexual recombination in the dataset. The hypothesis about random mating was rejected in cluster 2 by calculating the index of association I_A on clone uncorrected data ($I_A = 4.4$, $p < 0.01$). Similarly, sexual recombination was also rejected for the complete dataset of all *T. erinacei* samples based on microsatellite data ($I_A = 4.2$, $p < 0.01$; Fig. 7) and for the dataset where MLMT data were combined with MLST data (data not shown). The hypothesis about random mating was not rejected on clone-corrected data in either case (cluster 2 and the whole dataset) but not at the significance level. We observed a relatively high proportion of fixed microsatellite alleles in both clusters, as evidenced by the relatively high fixation index ($F_{ST} = 0.85$, $p < 0.000001$), which means that there is only limited genetic information exchange between the clusters.

Phylogeny and position of T. erinacei in the T. benhamiae complex

For the multigene phylogeny based on ITS, *gapdh*, *tubb* and *tef1-α* loci, we used previously published alignments (Čmoková et al. 2021) that were enriched by 71 *T. erinacei* isolates for which we generated sequences in this study. The final alignment included 443 strains representing the entire species diversity of the *T. benhamiae* complex. Detailed alignment characteristics for all phylogenies together with the partitioning schemes and substitution models are listed in Table 2. The isolation source and accession

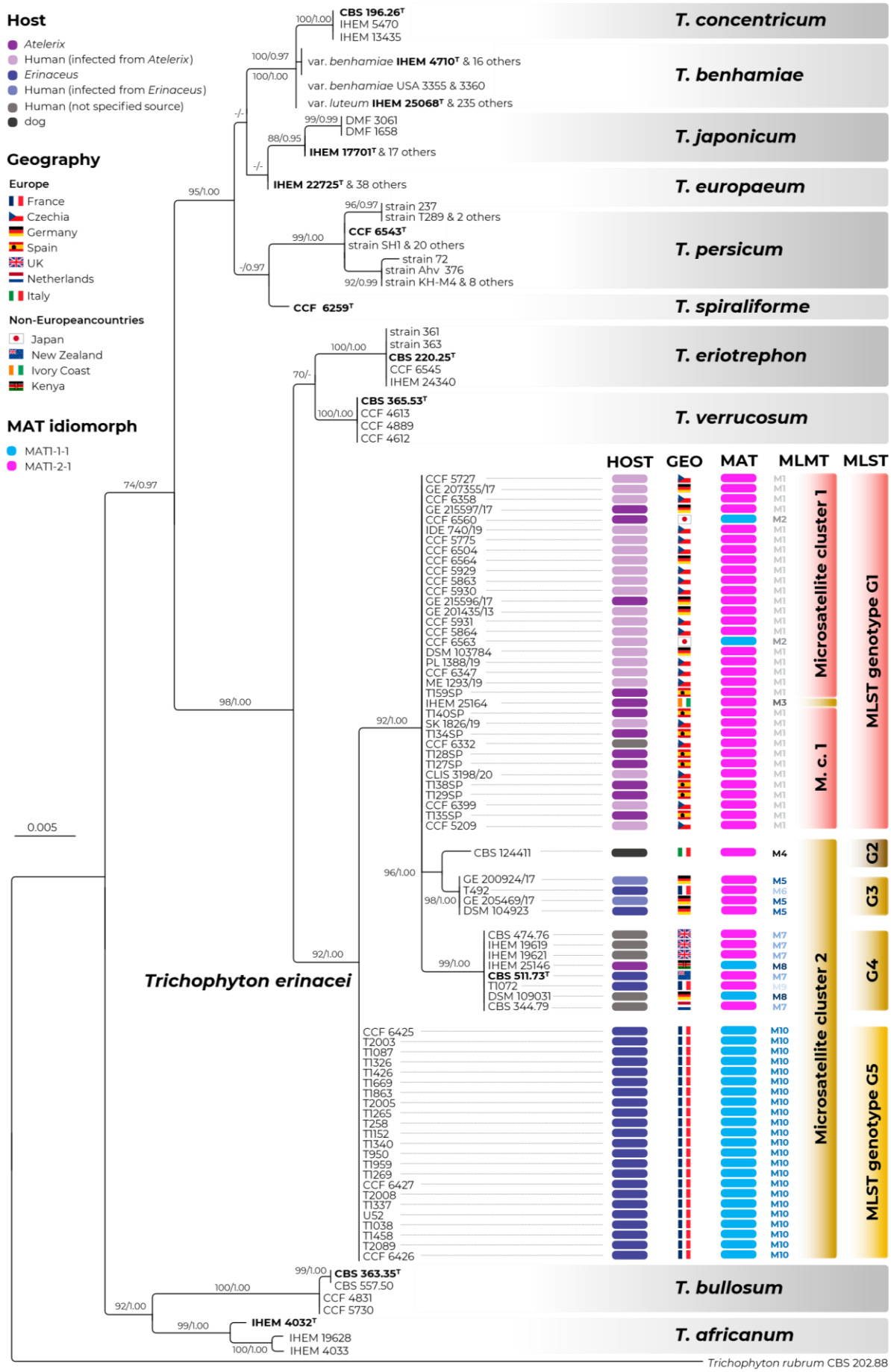
Host
Atelerix
Human (infected from Atelerix)
Erinaceus
Human (infected from Erinaceus)
Human (not specified source)
dog
Geography
Europe
France
Czechia
Germany
Spain
UK
Netherlands
Italy
Non-Europeancountries
Japan
New Zealand
Ivory Coast
Kenya
MAT idiomorph
MAT1-1-1
MAT1-2-1
0.005
CBS 196.26T
IHEM 5470
IHEM 13435
T. concentricum
var. benhamiae IHEM 4710T & 16 others
var. benhamiae USA 3355 & 3360
var. luteum IHEM 25068T & 235 others
T. benhamiae
DMF 3061
DMF 1658
IHEM 17701T & 17 others
T. japonicum
IHEM 22725T & 38 others
T. europaeum
strain 237
strain T289 & 2 others
CCF 6543T
strain SH1 & 20 others
strain 72
strain Ahv 376
strain KH-M4 & 8 others
T. persicum
CCF 6259T
T. spiraliforme
strain 361
strain 363
CBS 220.25T
CCF 6545
IHEM 24340
T. eriotrephon
CBS 365.53T
CCF 4613
CCF 4889
CCF 4612
T. verrucosum
HOST
GEO
MAT
MLMT
MLST
Microsatellite cluster 1
M. c. 1
MLST genotype G1
Microsatellite cluster 2
MLST genotype G5
G2
G3
G4
Trichophyton erinacei
T. bullosum
CBS 363.35T
CBS 557.50
CCF 4831
CCF 5730
T. africanum
IHEM 4032T
IHEM 19628
IHEM 4033
Trichophyton rubrum CBS 202.88

Fig. 3 Multilocus phylogeny of *Trichophyton benhamiae* complex inferred with the maximum likelihood method based on the ITS rDNA, *tef1-α*, *gapdh* and *tubb* loci (alignment characteristics, partitioning scheme and substitution models are listed in Table 2). A total of 71 *T. erinacei* isolates were characterized, included in the phylogeny, and the following information was plotted on the tree and designated by different colours: host from which isolate was obtained (column HOST), geographic origin (GEO), mating type gene (MAT) idiomorph, multilocus genotype based on 4 loci sequences (MLST; G1-G5), multilocus genotype based on seven microsatellite markers (MLMT; M1-M10) and assignment of isolates into cluster based on analysis in STRUCTURE software (Cluster 1 – yellow, Cluster 2 – red). Maximum likelihood bootstrap values and Bayesian posterior probabilities are appended to the nodes; only support values higher than 70% and 0.95, respectively, are shown. The ex-type strains are designated with a superscripted T and bold print. *Trichophyton rubrum* CBS 202.88 was used as the outgroup.

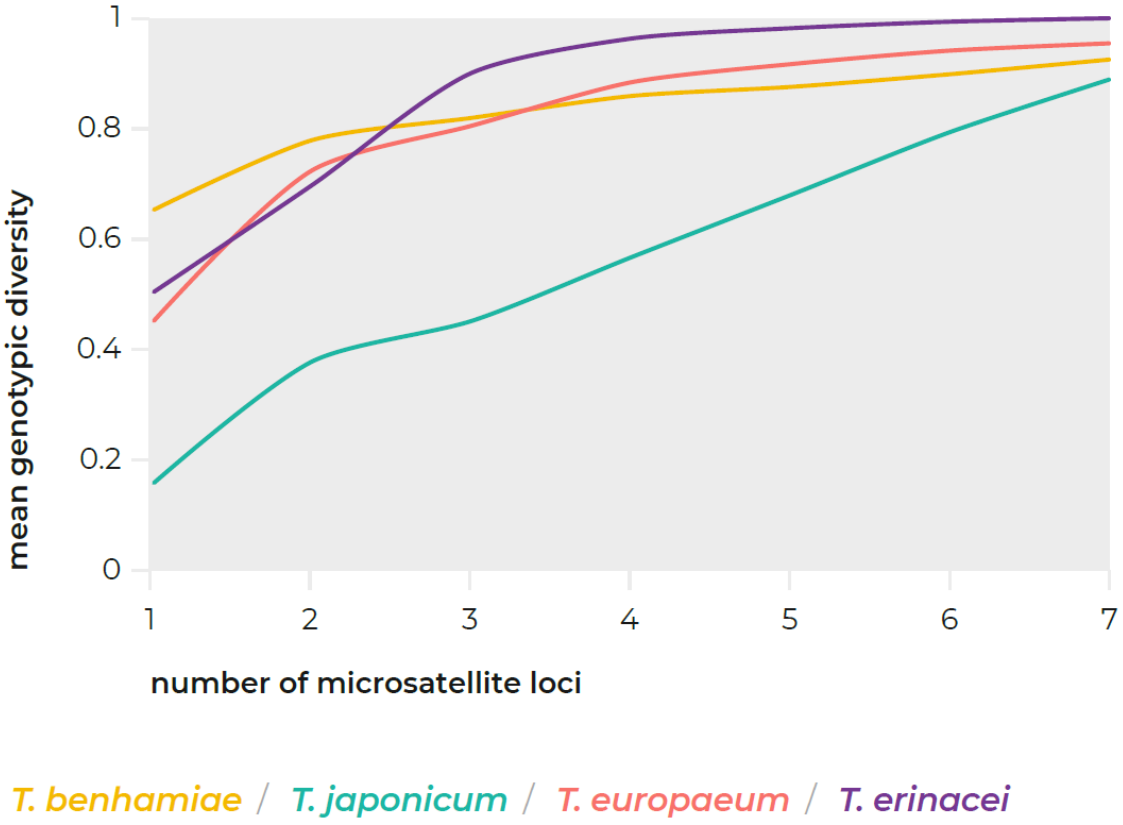

Fig. 4 Plot of mean genotypic diversity as a function of the number of micro- satellite loci.

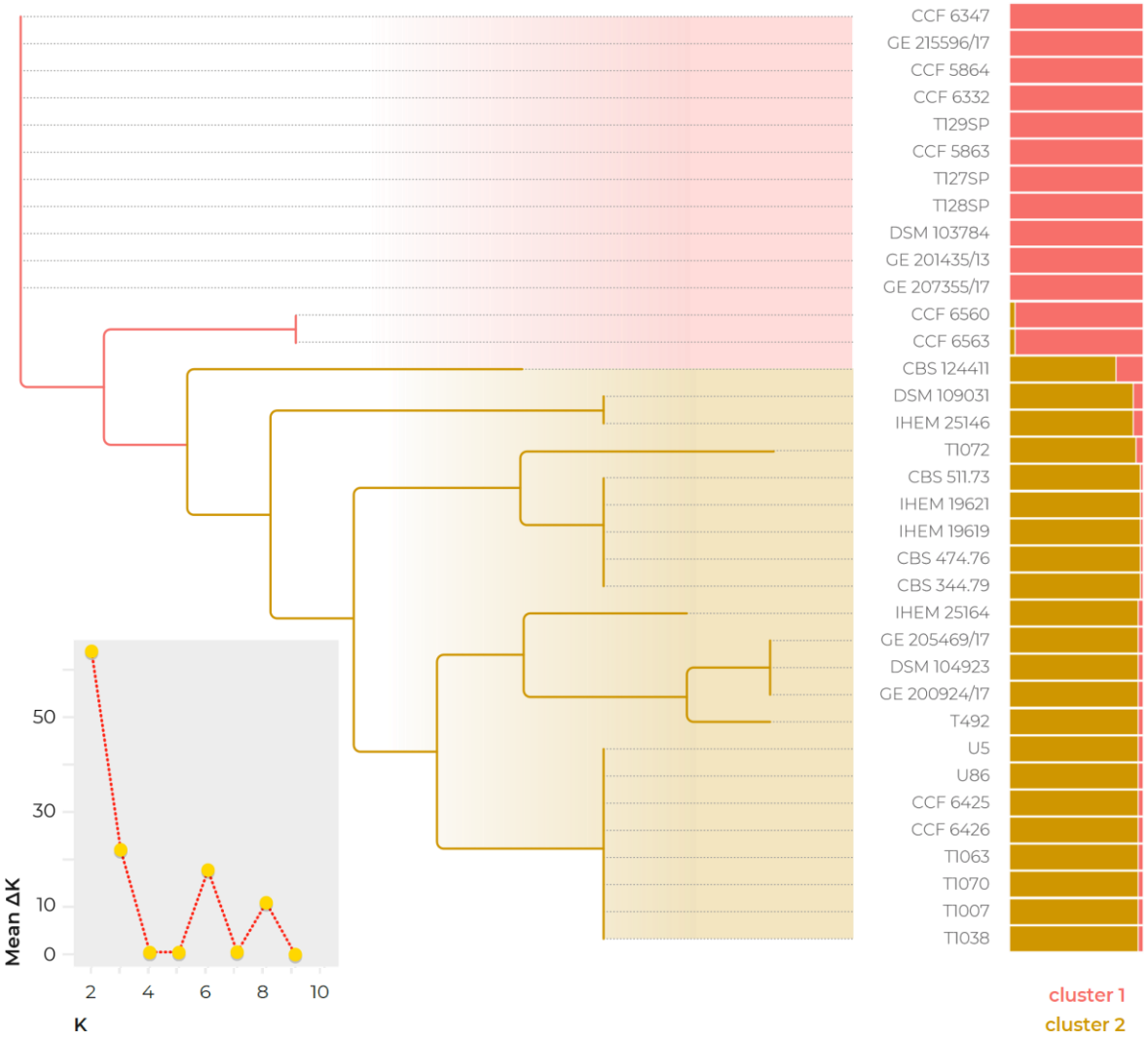

Fig. 5 Population structure plot for distribution of *Trichophyton erinacei* isolates into two clusters (K = 2 according to the peak of ΔK; graph is shown in the lower left corner of the figure) based on seven microsatellite loci. The plot created with STRUCTURE software using Bayesian clustering is displayed vertically on the right side of the Figure. Individual isolates (haplotypes) are represented by horizontal bars. Dataset was reduced to ≤5 samples from each locality to minimize effect of uneven sampling. A neighbour-joining tree calculated from the multilocus microsatellite profiles using the Jaccard distance matrix measure in FAMD software is used for the comprehensive presentation of the results.

numbers for DNA sequences are available in Table 1 and S1. The alignments were deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.pzgmsbcnv).

Single-gene phylogenies were highly incongruent, and genes with more genotypes did not resolve *T. erinacei* strains as a monophyletic group (Fig. 8). The isolates were divided into three lineages in the ITS phylogeny. Four strains from *Erinaceus* hedgehogs, corresponding to MLST genotype G3, did not

cluster with the majority of *T. erinacei* strains and were more closely related to *T. verrucosum* (Fig. 8). These strains had three unique substitutions in the ITS region and another three substitutions that were only shared with strain CBS 124411 (MLST genotype G2). The strain CBS 124411 was resolved on a long branch sister to the vast majority of *T. erinacei* strains (n = 66), and its ITS sequence contained five unique substitutions that were not shared with any other *T. erinacei* isolate (eight substitutions compared to the ex-type strain).

In the phylogeny based on the *tef1-α* gene, *T. erinacei* strains were divided into two groups. The majority of strains (n = 63) clustered with *T. verrucosum* and *T. eriotrephon*. Eight strains, corresponding to MLST genotype G4, also clustered with the species mentioned but were in a basal position to them. The *tef1-α* sequences of these strains associated with both *Erinaceus* and *Atelerix* hedgehogs contained 11 substitutions compared to the remaining *T. erinacei* strains.

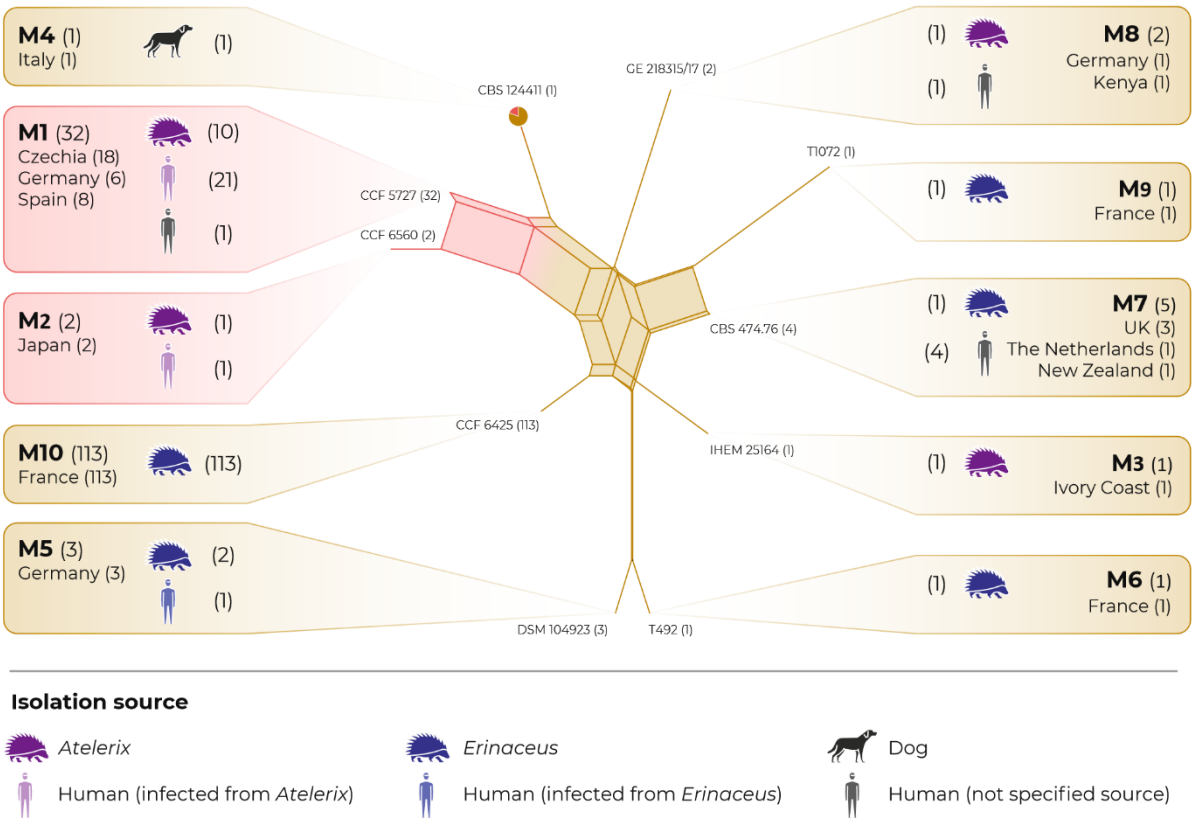

Fig. 6 Population structure of *Trichophyton erinacei* revealed by the analysis of seven microsatellite loci in 161 strains. The NeighborNet network was built with FAMD software and visualized in SplitsTree using the Jaccard index-based distance matrix. Geographic origin and host(s) are detailed separately for each multilocus genotype M1-M10.

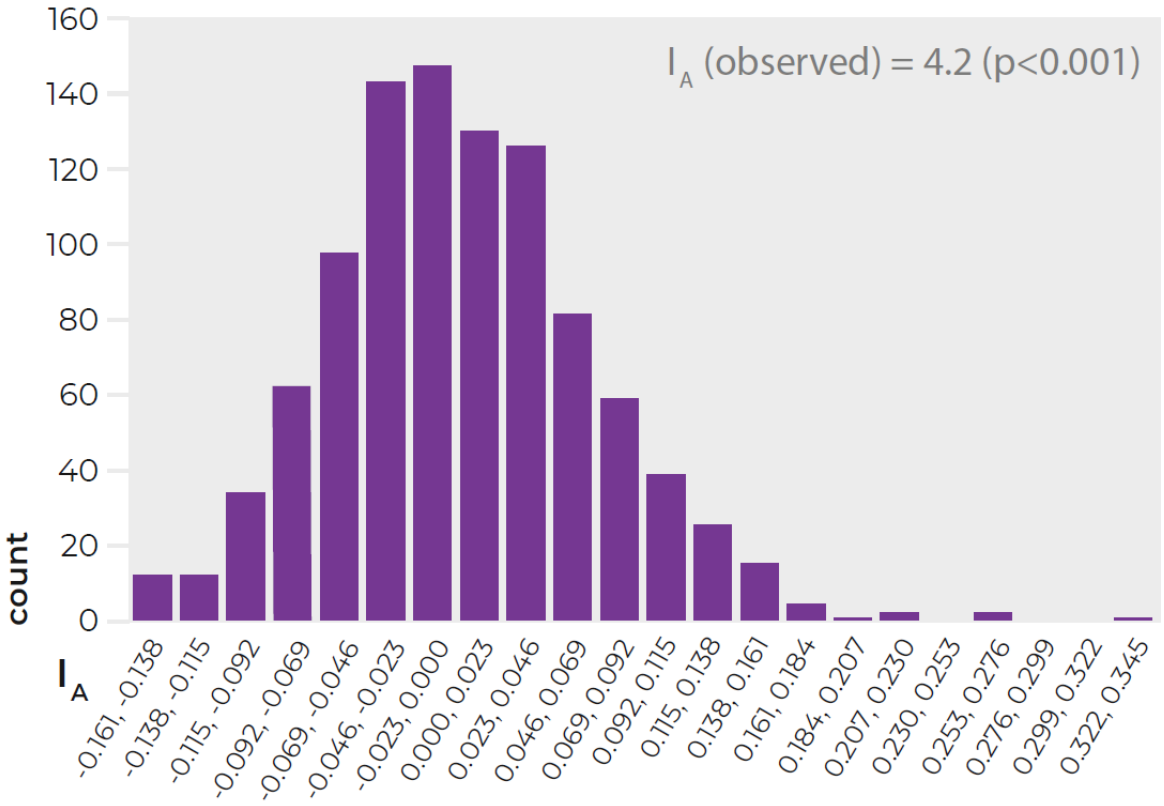

Fig. 7 Histogram of the simulated index of association (I_A) for Cluster 2 isolates from 10 000 permutations of randomization tests under a null model of allelic recombination; the observed value of I_A is 4.2 and is out of 95% confidence interval of the simulated values.

The phylogeny based on the *gapdh* gene also divided isolates into two considerably remote groups. The French isolates from hedgehogs of the genus *Erinaceus*, corresponding to MLST genotype G5, clustered with *T. verrucosum* and *T. eriotrephon*, while the remaining strains were resolved in the basal position to other *T. benhamiae* complex species. In total, these two groups differed by 11 substitutions in the *gapdh* gene. In contrast to single-gene phylogenies, the multigene phylogeny resolved *T. erinacei* as a monophyletic species (Fig. 3). Within the *T. benhamiae* complex, species were resolved into three major monophyletic clades, corresponding to *T. benhamiae*, *T. erinacei* and *T. bullosum* clades sensu Čmoková et al. (2020). *Trichophyton erinacei* formed an independent lineage within the *T. erinacei* clade, which also encompassed *T. verrucosum* and *T. eriotrephon*.

In addition to DNA sequence markers, we also constructed a phylogenetic network based on seven microsatellite markers across the members of *T. benhamiae* and *T. erinacei* clades (except for *T. verrucosum*). In this network shown in Fig. 9, all *T. erinacei* strains clustered together in agreement with

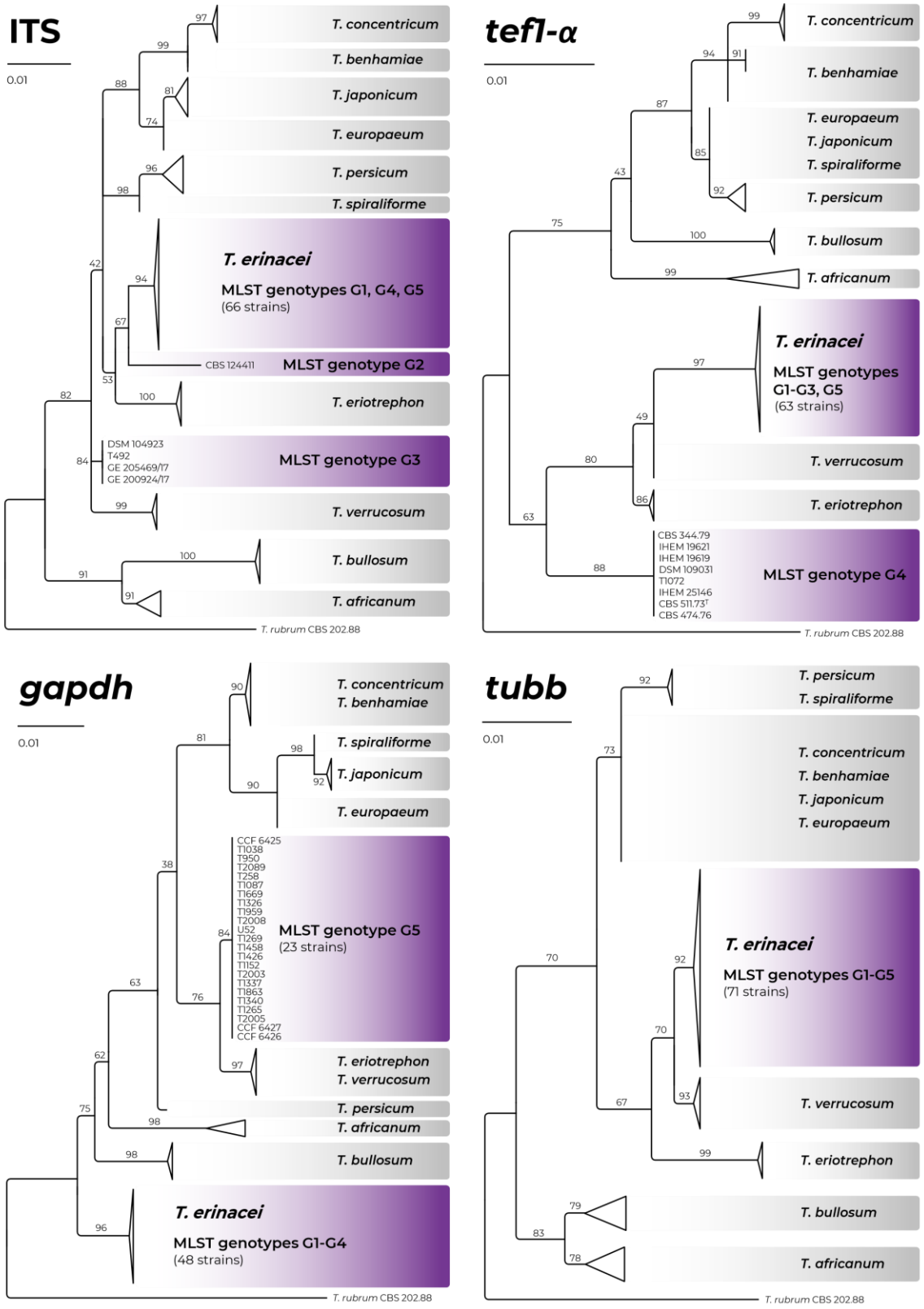

Fig. 8 A best scoring maximum likelihood tree based single genes: ITS rDNA, *tef1-α*, *gapdh* and *tubb* substitution models are listed in Table 2). *Trichophyton erinacei. Trichophyton erinacei* strains are highlighted with violet colour; corresponding multilocus genotypes (MLST) from the multiple gene phylogeny (Fig. 3) are listed. Maximum likelihood bootstrap values are appended to the nodes; *Trichophyton rubrum* CBS 202.88 was used as the outgroup.

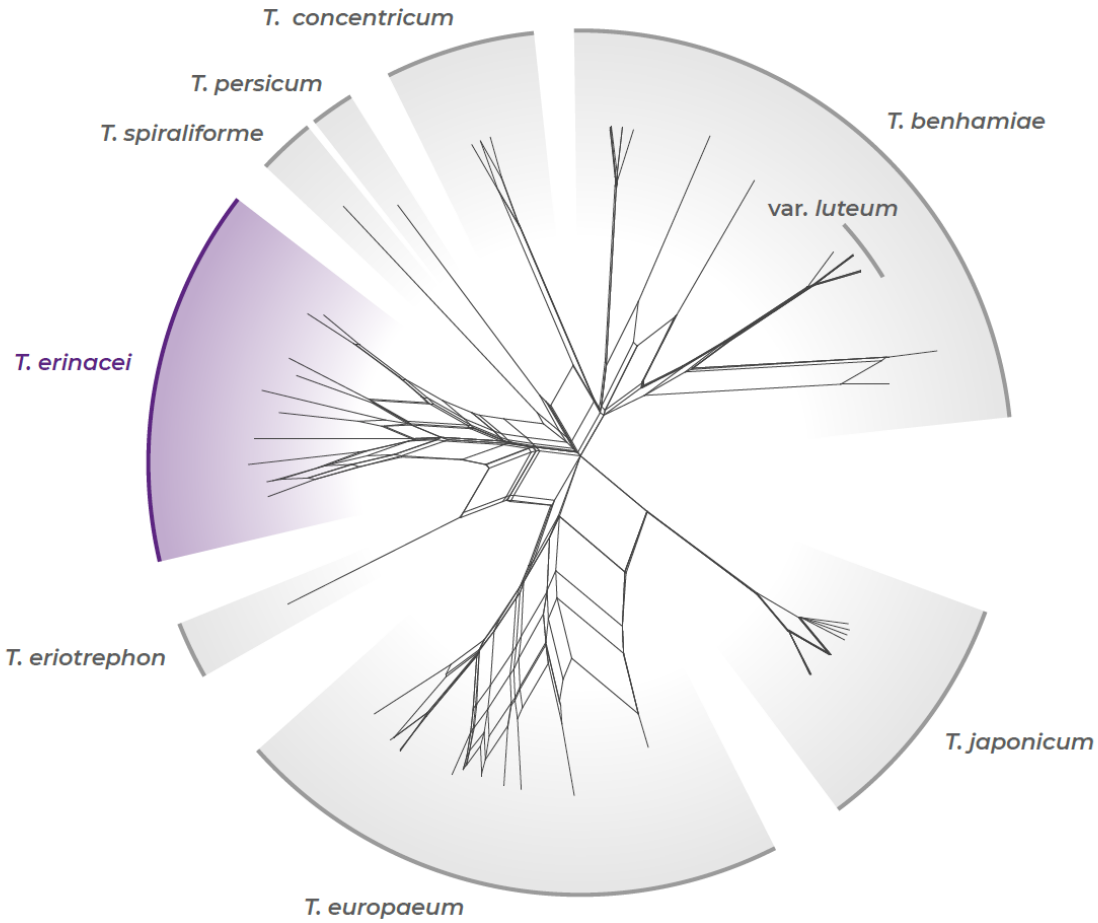

Fig. 9 Population structure of the *T. benhamiae* complex revealed by the analysis of seven microsatellite loci in 483 strains (Table S1). *Trichophyton verrucosum*, *T. bullosum* and *T. africanum* were not included because the markers could not be amplified in them. The NeighborNet network was built with FAMD software and visualized in SplitsTree using the Jaccard index-based distance matrix. *Trichophyton erinacei* strains are indicated by violet colour.

the results of the multigene phylogeny. The relationships between species resulting from this analysis were also similar to phylogeny (Fig. 3). *Trichophyton eriotrephon* was the most closely related species to *T. erinacei*, followed by a group of species from the *T. benhamiae* clade.

Phenotypic analysis

All *T. erinacei* stains were initially examined on MEA at 25 °C. For more detailed morphological examination, at least two strains (if available) of each MLST genotype were selected and cultivated on SGA, MEA and PDA media at 25 °C for 7 d with a subsequent measurement of micromorphological characteristics after 2 wk of cultivation. In total, 26 strains were examined.

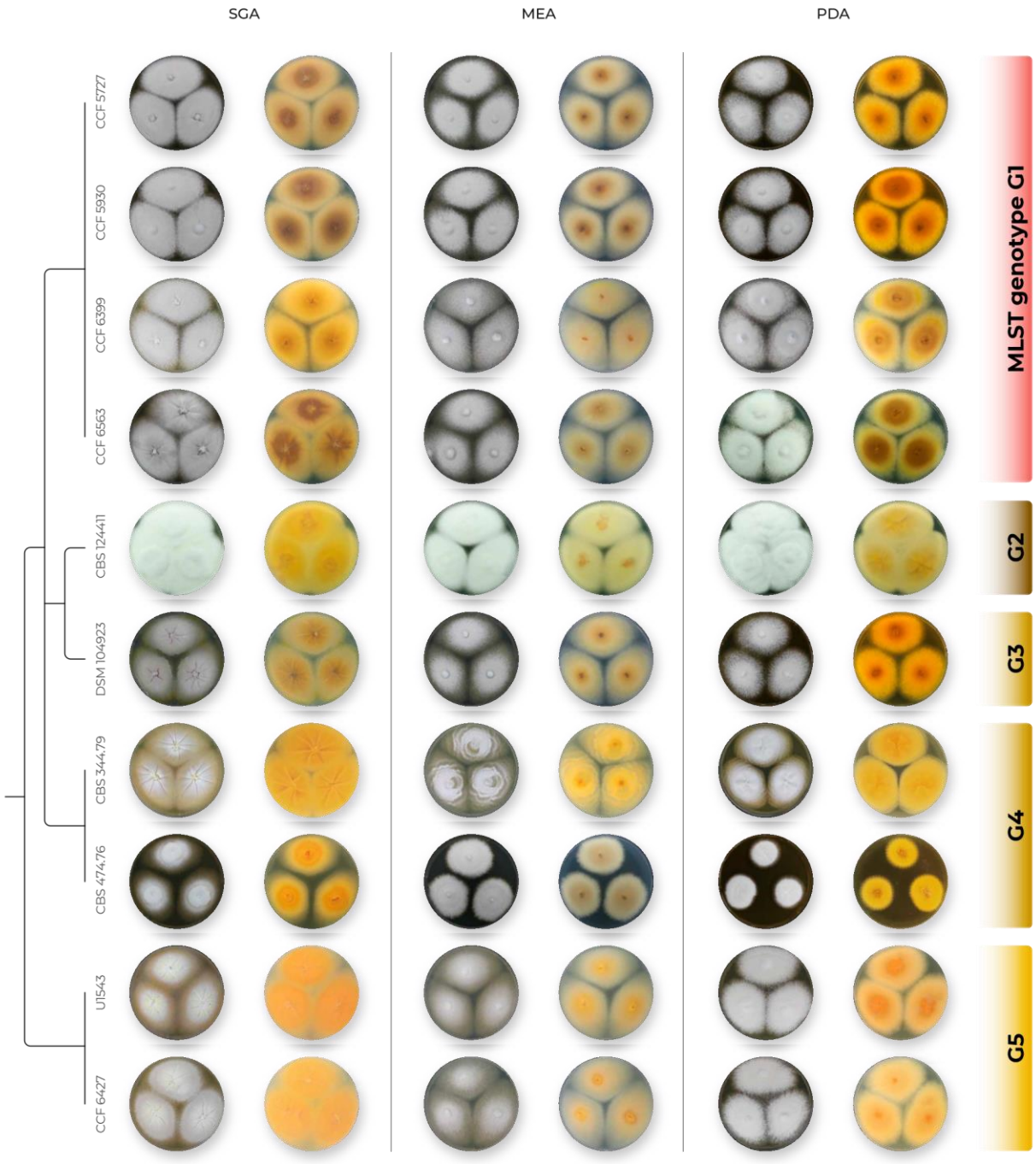

Fig. 10 Overview of the macromorphology of the *Trichophyton erinacei* isolates representing different multilocus genotypes. The isolates were cultivated on Sabouraud dextrose agar (SAB), malt extract agar (MEA) and potato dextrose agar (PDA) for 14 days at 25 °C; obverse and reverse of colonies is shown.

There were no statistically significant differences in the growth rates or visible differences in the macromorphology of colonies (Fig. 10), which could be linked to MLST genotypes or microsatellite clusters. Colonies of strains from cluster 1 reached 22–26 mm diam (Ø = 24 mm) on MEA, 23–35 mm

diam (Ø = 26 mm) on SGA and 17–32 mm diam (Ø = 21 mm) on PDA in 7 d at 25 °C. Colonies of strains from cluster 2 reached 19– 45 mm diam (Ø = 28 mm) on MEA, 21–43 mm diam (Ø = 29 mm) on SGA, and 11–35 mm diam (Ø = 24 mm on PDA in 7 d at 25 °C. In particular, the strains isolated before 2000 showed cottony colonies and poor sporulation and thus could not be properly examined. Typical strains were characterized by finely to coarsely granular or powdery colonies that were flat, centrally raised or umbonate. Colony colour varied in individuals from white (#F2F3F4) to yellowish white (#F0EAD6). The reverse of colonies was pale-yellow (#F3E5AB) or brilliant yellow (#FADA5E) on all examined media after 14 d of cultivation. In some strains, the reverse colour in the centre of colonies turned brown (Fig. 10).

microconidia on all media compared to cluster 2 isolates (n = 15), which did not sporulate so intensively, possibly a consequence of the slightly different arrangement of conidiophores. Conidiophores of cluster 1 strains showed mostly a pyramidal (grape-like) arrangement and therefore tended to produce larger quantities of spores, while conidiophores of cluster 2 were less extensively branched with conidia mostly sessile or borne along the conidiogenous hyphae on short protrusions or short lateral branches. The size and shape of microconidia also differed between clusters. Microconidia of cluster 1 strains were significantly wider and shorter (Fig. 11) than microconidia of cluster 2 ($p < 0.0001$) and were rather pyriform, 2.2–7.4 (3.9 ± 0.7) × 1.4–3.5 (2.3 ± 0.3) µm, while conidia of cluster 2 were predominantly clavate, 2.5–8.7 (4.2 ± 0.8) × 1–3.4 (2.1 ± 0.3) µm.

Although sizes were significantly different, there was a large overlap in the dimensions, and significant interindividual differences between strains from the same cluster were detected. Therefore, the observed differences could not be used in the differentiation of clusters in practice. Macroconidia were absent in most *T. erinacei* isolates. When present, they were rather sparse to rare, cigar-shaped or clavate, with a tapering rounded apex and truncate base, and usually consisted of 2–8 cells (median 4), 25.5–33.5 (26.8 ± 3.5) × 4.3–6 (5 ± 0.6) µm. Chlamydospores were rarely present. No spiral hyphae were found even after 21 d of incubation. Strain CBS 474.46, ex-type of *T. proliferans*, was characteristic by more intensive production of macroconidia and poor production of microconidia. The shape of its macroconidia was also different from the shape of macroconidia of the other examined strains, which were thin and long or flask-shaped, with pointed ends, and consisted of 2–6 cells (median 4), 10–72.5 (39 ± 19) × 2.5–5.5 (3.5 ± 1) µm. These macroconidia are often produced intercalarily in vegetative hyphae.

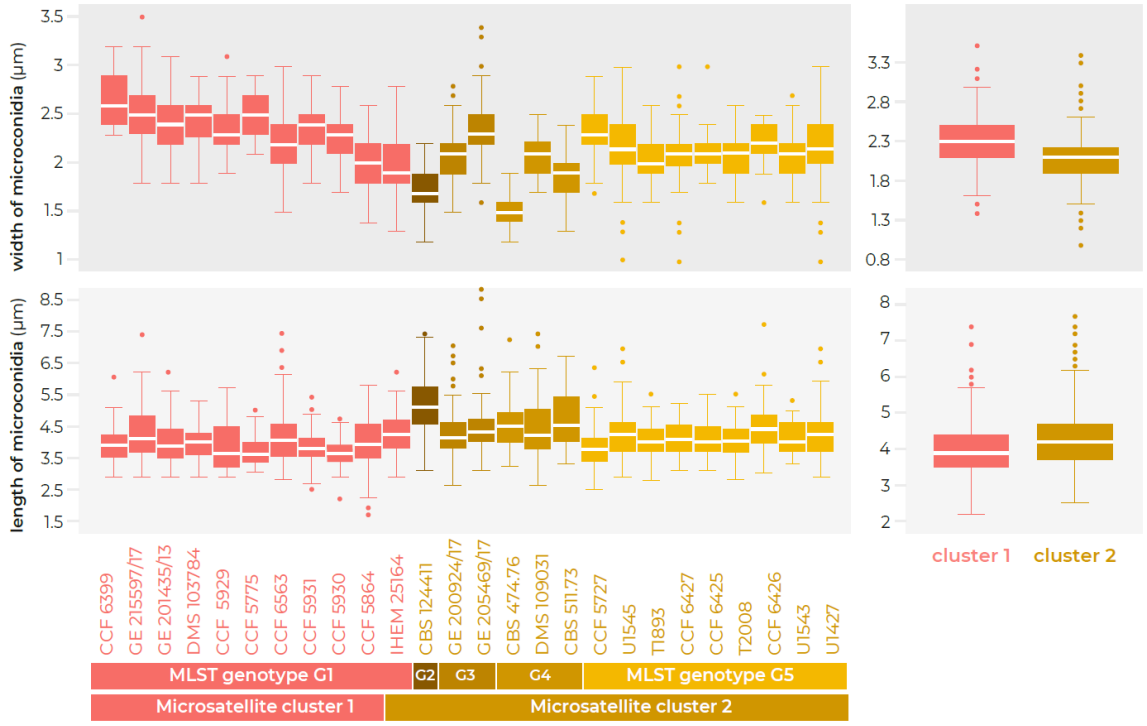

Fig. 11 Length and width of microconidia in Trichophyton erinacei isolates representing different microsatellite clusters/multilocus genotypes (MLST). The horizontal lines in boxplots indicate the mean value and interquartile range, whiskers span the 5 % and 95 % percentiles, and dots indicate extreme outliers.

Clinical picture and epidemiology

Clinical and epidemiological data associated with strains examined in this study are summarised in Table 3. The spectrum of patients and clinical manifestations of human and animal infections due to *T. erinacei* did not differ between the two clusters. The human patients were within the range of 2–46 yr (median 23 and 26 yr, respectively) and were predominantly women (75 and 86 %, respectively). Infections usually manifested as tinea corporis (Fig. 2) localised on the upper extremities (predominantly hands), face and trunk. Infections in animals (mostly young individuals) were frequently asymptomatic or manifested as scaly skin or spine loss (Table S1).

Table 3 Comparision of clinical and epidemiological data associated with strains representing different populations of *Trichophyton erinacei* (cluster 1 and cluster 2)

Source	Clinical data	Population[1]	
		cluster 1	cluster 2
Human	Number of samples	23	7
	Sex	Women 86 %	Women 75 %
	Age range (median)	15-46 (26)	2-28 (23)
	Site of infection	Upper extremities (59 %), face (13 %), trunk (13 %)	Upper extremities (100 %)
	Contracted from: *Erinaceus* / *Atelerix* / unknown	0 / 22 /1	2 / 0 / 5
Animal	Number of samples	11	120
		0 / 11 / 0	117 / 2 / 1
Total number of samples		34	127

[1] Delimited by STRUCTURE software.

Most human patients infected with isolates from cluster 1 (~ 96 %, 22 out of 23) reported contact with pet hedgehogs of the genus *Atelerix*. The animal strains from cluster 1 were all isolated from *Atelerix* hedgehogs (n = 11). Most isolates (~ 80 %, n = 101 of 127) belonging to cluster 2 were obtained from European hedgehogs (*Erinaceus* spp.) or were isolated directly from *Erinaceus* hedgehogs (n = 2). Cluster 2, however, also contained two samples from wild African hedgehogs of the genus *Atelerix* from Africa.

Antifungal susceptibility

The antifungal susceptibility to tavaborole, ciclopirox and terbinafine differed significantly (Fig. 12) between isolates from cluster 1 (n = 10) and cluster 2 (n = 14), while the results from the remaining antifungal drugs (amorolfin, griseofulvin, luliconazole, itraconazole and efinaconazole) showed no significant differences.

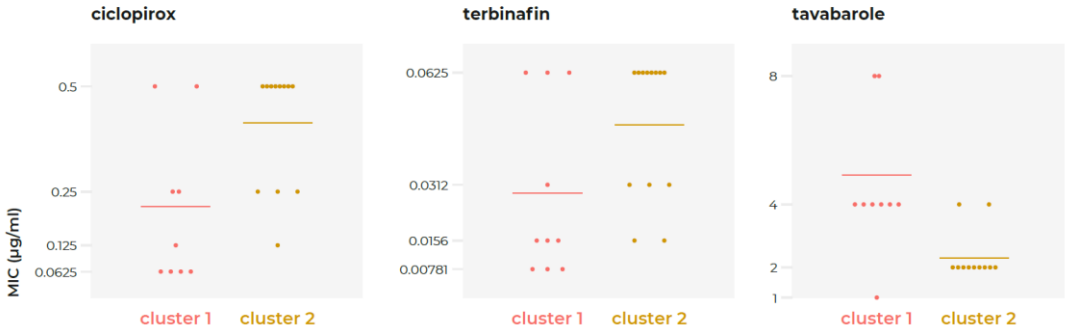

Fig. 12 Differences in antifungal susceptibilities in Trichophyton erinacei isolates from cluster 1 and cluster 2. Significant differences in susceptibility to ciclopirox, terbinafine and tavaborole were found (ANOVA, $p < 0.05$) while no statistically significant differences were found in susceptibility to the remaining five antifungals (see Results). The dots in the graph represent susceptibilities of individual isolates and geometric mean values are indicated by horizontal lines.

Cluster 1 strains were significantly less susceptible to tavaborole, with MICs ranging between 1–8 µg/mL (median 4) than cluster 2 strains (2–4 µg/mL; median 2) (ANOVA, $p < 0.001$). In contrast, cluster 1 strains were more susceptible to ciclopirox with lower MICs (0.0625–0.5 µg/ mL; median 0.1875) than the MICs from cluster 2 strains (0.125–0.5 µg/mL; median 0.5) (ANOVA, $p < 0.005$). Similarly, cluster 1 strains were more susceptible to terbinafine (MICs 0.00781–0.0625 µg/mL; median 0.0156) than cluster 2 strains (0.0156–0.0625 µg/mL; median 0.0625) (ANOVA, $p < 0.05$).

DISCUSSION

Hedgehogs have become fashionable pets in many countries worldwide. These animals are a potential source of many pathogens with zoonotic potential, including bacteria, viruses and fungi (Riley & Chomel 2005, Ruszkowski et al. 2021). Despite these facts, hedgehog-borne infections are generally of low public awareness. Together with the high prevalence of *T. erinacei* in hedgehogs, this low public awareness contributes to a significant increase in reported cases in recent years. Despite the growing importance of *T. erinacei* as a cause of zoonotic dermatophytoses, little is yet known about its taxonomy and diversification of populations depending on the different hedgehog hosts and the geographical origin of isolates. In this study, we collected the largest set of isolates from different hosts to date and examined population genetics using 11 genetic markers to address the questions mentioned.

Based on microsatellite data, the isolates of *T. erinacei* are distributed into two main subpopulations (clusters 1 and 2) with limited gene flow between them. The random mating hypothesis was rejected (I_A) for the whole species *T. erinacei* as well as for cluster 2 which is more diversified than cluster 1. However, this does not mean that there are no occasional recombination events between strains within and between subpopulations, rather reflecting that isolates examined in the present study spread clonally between hosts from which they were isolated. Most notably, isolates belonging to cluster 1 (MLMT genotypes 1 and 2 / MLST genotype G1) spread clonally between pet African hedgehogs, while cluster 2 strains spread predominantly between *Erinaceus* wild hedgehogs. The isolates of cluster 2 were more diverse in terms of genetic variability and ecology. Most of the isolates were obtained from European hedgehogs (*Erinaceus*) admitted to wildlife rehabilitation centres in France (Le Barzic et al. 2021), and except for two strains (T1072 and T492), all belonged to a single clone (MLMT genotype 10 / MLST genotype G5). The remaining minority of strains of cluster 2 were obtained from both *Erinaceus* and *Aleterix* hedgehogs from different countries and belonged to diverse MLST and MLMT genotypes (Fig. 3, 6).

From a genetic point of view, we can observe several signs of sexual reproduction/recombination in the population of *T. erinacei*. First, both mating type genes were present among isolates representing both clusters. Second, we observed incongruences between DNA sequence data and microsatellite data, and some isolates had significant admixture levels between microsatellite clusters (CBS 124411; Fig. 5). The intraspecific genetic variability in *T. erinacei* is higher than the intraspecific genetic variability in

other species of the *T. benhamiae* complex (Čmoková et al. 2020, 2021). Although the number of MLMT genotypes was comparable to the number of MLMT genotypes of *T. europaeum*, this species showed no variability in the DNA sequence data. A relatively high number of MLST genotypes detected among *T. erinacei* strains have also been observed in another sexual dermatophyte species, *T. mentagrophytes*, which also includes isolates of both mating types (Suh et al. 2018, Tang et al. 2021). Specific MLST genotypes in *T. erinacei* are substantiated by a relatively large number of substitutions that are present in certain strains, contributing to the polyphyletic nature of *T. erinacei* in the single-gene phylogenies (Fig. 8) and highlighted the pitfalls of constructing phylogeny in species groups where both sexual (*T. erinacei*) and clonal (e.g., *T. verrucosum* and *T. eriotrephon*) species are present. In general, the observed incongruence between single gene genealogies could be explained by past recombination/hybridization events or sharing of an ancestral polymorphism caused by incomplete lineage sorting, typical for recently diverged species (Hubka et al. 2018a, Steenkamp et al. 2018, Matute & Sepúlveda 2019, Kandemir et al. 2020). However, both the multigene phylogeny and network constructed from multilocus microsatellite data resolved *T. erinacei* as a monophyletic species (Fig. 3, 9).

In summary, we detected some level of substructuring into two subpopulations in *T. erinacei* that are associated with different hedgehog genera. These populations are not completely separated and there is a certain gene flow between them, probably due to the occasional rare transmission of pathogen between different hedgehog hosts. We also found minor differences in the micromorphology and antifungal susceptibilities between these two subpopulations, which are not large enough to recognise them as separate taxonomic entities, also supported by a coherent ecology strongly linked to hedgehogs and by similar growth parameters of strains belonging to different populations.

The soil environment enriched by keratin sources is necessary for the sexual reproduction of dermatophyte species (Weitzman & Summerbell 1995, Tang et al. 2021). According to this theory, anthropophilic and some zoophilic dermatophytes lost their sexuality due to lack of contact with soil habitat. Only some zoophilic *Trichophyton* species are believed to retain their ability to reproduce sexually, namely, *T. africanum*, *T. benhamiae*, *T. erinacei* and *T. mentagrophytes* (Hubka et al. 2018b, Čmoková et al. 2020, Metin & Heitman 2020, Tang et al. 2021). As far as we know, *T. erinacei* has not been cultivated directly from soil. However, its ability to survive in soil for more than one year was described by English & Morris (1969). Therefore, contaminated soil or hedgehog nests seem likely to be a source of infection to humans, dogs and other animals (English et al. 1962, Quaife 1966, English & Morris 1969, Romano et al. 2001).

Wild hedgehogs are in close contact with soil where sexual reproduction should take place and where genetic variability is expected to be generated by recombination. Therefore, we expected to find considerable genetic variability among 115 isolates from European hedgehogs from France (in the proximity of the Paris region) (Le Barzic et al. 2021). Surprisingly, this set of strains consisted mostly of clonal isolates (MLMT genotype 10) with *MAT1-1-1* gene idiomorph and only two strains, T1072

and T492, represented different genotypes and had an opposite mating type gene idiomorph, indicating that the sexual reproduction and de novo infection of hedgehogs from soil is a rare event and that clonal spread secured by horizontal spread from individual to individual strongly dominates. This spread occurs through asexual arthrospores on hairs or in skin scales.

Future population genetic studies should focus on collecting more strains from localities that were underrepresented in our dataset such as isolates from wild African hedgehogs of the genus *Atelerix*, as they seem to represent a connecting link between isolates of clusters 1 and 2. Two African strains were included in this study, IHEM 25164 from the Ivory Coast (Gregory et al. 1978) and IHEM 25146 from Kenya (Gregory & English 1975). Strain IHEM 25164 showed a conflicting classification in terms of various molecular markers – MLST genotype G1 typical for cluster 1 but the strain was placed in cluster 2. Strain IHEM 25146 was the only verified isolate from the genus *Atelerix* among strains of cluster 2. Encompassing more samples from wild African hedgehogs would probably result in a better understanding of the population structure and gene flow between subpopulations of *T. erinacei*. Additionally, future collections of strains should be performed in collaboration with zoologists to clearly identify the host at the species level. For instance, European and African hedgehogs sampled in hybrid zones may be either *E. europaeus* or *E. roumanicus* and *A. albiventris* or *A. algirus*, or respective hybrids between mentioned pairs of species. Clear identification of hosts would allow us to study the extent to which the pathogen is transmitted between hedgehog species and how the ecological barrier of the host species may influence the population genetics of the pathogen.

The molecular markers used in this study represent powerful tools for surveillance of infections due to *T. erinacei* and provide for the first-time insight into the population structure and spreading strategy of this emerging pathogen. As the popularity of hedgehogs as pet animals increases, an increasing number of human infections can be expected in the future. The spread of these infections underscores the need for closer collaboration between veterinarians, dermatologists, epidemiologists, and public health personnel to set up appropriate preventive measures.

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Declaration on conflict of interest The authors declare that there is no conflict of interest.

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Supplementary material

Table S1 List of *Trichophyton* strains used in the phylogenetic analysis and population genetic analyses.

Supplementary material is available for download here:

PAPER 5

In vitro antifungal susceptibility patterns of the complex

Shamsizadeh F, Ansari S, Zarei Mahmoudabadi A, Hubka V, **Čmoková A**, Guillot J, Rafiei A, Zomorodian K, Nouripour-Sisakht S, Diba K, Mohammadi T, Zarrinfar H, & Rezaei-Matehkolaei A.

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In vitro antifungal susceptibility patterns of *Trichophyton benhamiae* complex isolates from diverse origin

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ABSTRACT

Background: Species from the *Trichophyton benhamiae* complex are mostly zoophilic dermatophytes which cause inflammatory dermatophytosis in animals and humans worldwide.

Objectives: This study was purposed to (a) to identify 169 reference and clinical dermatophyte strains from the *T. benhamiae* complex species by molecular method and adhering to the newest taxonomy in the complex (b) to evaluate the in vitro antifungal susceptibility profile of these strains against eight common and new antifungal agents that may be used for the treatment of dermatophytosis.

Methods: All isolates, mainly originated from Europe but also from Iran, Japan and USA, were subjected to ITS-rDNA sequencing. The in vitro antifungal susceptibility profiles of eight common and new antifungal drugs against the isolates were determined by CLSI M38-A2 protocol and according to microdilution method.

Results: Based on the ITS-rDNA sequencing, *T benhamiae* was the dominant species (n = 102), followed by *T. europaeum* (n = 29), *T. erinacei* (n = 23), *T. japonicum* (n = 10), *Trichophyton* sp. (n = 4) and

T. eriotrephon (n = 1). MIC ranges across all isolates were as follows: luliconazole: 0.0002–0.002 μg/ml, terbinafine: 0.008–0.125 μg/ml, efinaconazole: 0.008–0.125 μg/ml, ciclopirox olamine: 0.03–0.5 μg/ml, itraconazole: 0.06–2 μg/ml, griseofulvin: 0.25–4 μg/ml, amorolfine hydrochloride: 0.125–4 μg/ml and tavaborole: 1–16 μg/ml.

Conclusion: Luliconazole, efinaconazole and terbinafine were the most potent antifungals against *T benhamiae* complex isolates, regardless of the geographic locations where strains were isolated. These data might help dermatologists to develop effective therapies for successful treatment of infections due to *T. benhamiae* complex species.

KEYWORDS: Antifungal susceptibility, *T. benhamiae*, *T. erinacei*

INTRODUCTION

Trichophyton benhamiae complex traditionally encompasses six species, that is *T. benhamiae*, *T. bullosum*, *T. erinacei*, *T. eriotrephon, T. concentricum* and *T. verrucosum*.[1] Using polyphasic approach, *T benhamiae* was shown to be a complex of several zoophilic species resulting in proposal of three new species and one new variety, that is *Trichophyton benhamiae* var. *luteum* ('yellow phenotype' strains), *T. europaeum*, *T. japonicum* and *T. africanum*.[2] *Trichophyton benhamiae* was clinically considered less important in last decades but, after 2010, a suddenly high incidence of infections by this type of dermatophyte, from various European countries, that is Germany, Czech Republic and France was reported.[3-5] Dermatophytosis due to *T. benhamiae* usually affects young pet guinea pigs but it has also been reported in dogs, rabbits, cats, North American porcupines and some small rodents.[2] Zoonotic transmission is frequent and it can lead to highly inflammatory dermatophytosis of glabrous skin and scalp and less frequently nail infection.[6] Similarly, the hedgehog- associated species, *T. erinacei*, may also be considered as an emerging pathogen, as its incidence in animals and humans has obviously increased in the last decade.[4,7]

Traditionally, antifungal resistance has mainly been reported in fungi responsible for invasive infections. However, it is an emerging phenomenon in the case of dermatophytes, as well.[8-10] High level of in vitro terbinafine (TRF) resistance with known and novel mutations in squalene epoxidase (*SQLE*) gene in *T. mentagrophytes* isolates[11] in correlation to clinical treatment failure[12] have recently been reported in several Asian and European countries. Regular monitoring of antifungal susceptibility testing (AFST) in dermatophyte is helpful to detect the potential development of resistance and contribute towards finding appropriate antifungal therapy. Until now, no large- scale investigation has been conducted about in vitro activity of antifungal agents against *T. benhamiae* complex isolates. The present study evaluates the in vitro antifungal susceptibility of a large set of human and animal clinical *T. benhamiae* complex isolates against terbinafine, griseofulvin, itraconazole, efinaconazole, luliconazole, amorolfine hydrochloride, ciclopirox olamine and tavaborole.

MATERIAL AND METHODS

Isolates

A collection of reference strains and clinical isolates from *T. benhamiae* complex (*n* = 169) were examined in the study (Table 1). The isolates mainly originated from Europe but also from Iran, Japan and USA. Majority of isolates (*n* = 164) were already included in the study by Čmoková et al[2] In cases with available information regarding the source (human or animal) and clinical type of infection, data were used for analysis (see below). The ethic approval for the study was provided by the Research Ethic Board of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (approval ID: IR.AJUMS. REC.1396.912).

Molecular identification

All isolates were subjected to sequencing of the internal transcribed spacer regions of ribosomal DNA (ITS- rDNA).[1] Briefly, each isolate was sub- cultured on Sabouraud glucose agar with chloramphenicol and cycloheximide (SDA; BD Diagnostics) and incubated at 28°C till enough growth. The DNA was extracted by mechanical disruption of mycelium growth in a homogenising device (SpeedMill PLUS; Analytik Jena) as described previously,[13] then purified and precipitated with phenol- chloroform-isoamyl alcohol (25:24:1) and ethanol, respectively. The Bio- Rad C1000 Touch thermal cycler (Bio-Rad) was programmed for amplification of ITS- rDNA region by the primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG- 3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC- 3′)[14] as initial denaturation at 94°C for 6 min, then 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min followed by 10 min final extension at 72°C. The amplified products from this amplification were purified and bidirectionally sequenced with the BigDye Terminator sequencing mix (Applied Biosystems) in a DNA sequencer (ABI Prism 3730XL; Applied Biosystems). The obtained sequences were uploaded into MEGA software ver. 7.0,[15] checked for quality and aligned. Finally, the aligned ITS- rDNA sequences were compared with the reference sequences of the *T benhamiae* complex ex- type strains deposited in GenBank, and then identified at the species level (Table 1).

Antifungal susceptibility testing

A panel of eight common and new topical or systemic antifungal agents was tested against *T. benhamiae* complex isolates by the Clinical and Laboratory Standards Institute broth microdilution method, according to CLSI M38- A2 document for moulds.[16] The antifungal drugs were as follows: terbinafine (TRB; Combi- Blocks Inc.,), griseofulvin (GRZ; Sigma- Aldrich), itraconazole (ITC; Sigma- Aldrich, Darmstadt, Germany), efinaconazole (EFN; Sigma- Aldrich), luliconazole (LUL; Sigma- Aldrich), amorolfine hydrochloride (AMF; LKT Laboratories Inc.,), ciclopirox olamine (CPO; LKT Laboratories Inc.,) and tavaborole (TVB; Sigma- Aldrich). Stock solution of each antifungal agent was prepared in dimethyl sulfoxide (DMSO) and diluted in RPMI 1640 medium with L- glutamine but without sodium

bicarbonate (Gibco) and buffered with morpholinepropanesulfonic acid (MOPS; Bio Basic Inc.,) in concentration twice the final concentration in sterile 96-well microplates. The final concentration of each antifungal agent ranged as follows: 0.001- 0.5 µg/ml for TRB, 0.015- 8 µg/ml for GRZ, 0.016- 8 µg/ml for ITC, 0.001 to 0.5 µg/ml for EFN, 0.00006- 0.031 µg/ml for LUL, 0.03- 16 µg/ml for AMF and 0.004- 2 µg/ml for CPO, and 0.03- 16 µg/ml for TVB.

To induce conidiation, isolates were cultured for up to 14 days at 28°C on 10 cm Petri plates containing 10- fold diluted Sabouraud Glucose agar.[17] To provide conidial suspension, mature colony was submerged with sterile saline containing 0.05% (w/v) Tween 20 and the surface was rubbed with a sterile scraper. The resulting mixture was then transferred into 5- ml sterile tubes and was allowed to sediment for 20 min.

Table 1 Distribution of *Trichophyton benhamiae* complex Isolates in this study regarding species, geographic location and type of tinea infection

Species	Number of clinical/ standard isolates	Country of origin	Clinical manifestation	Molecular identifaction by ITS-rDNA (accession number)
T. benhamiae	102	Belgium, Czech Republic, France, Iran, Japan, Switzerland, USA	tinea corporis (faciei) tinea capitis	100% similarity with ex-type IHEM 4710 (LR794129)
T. europaeum	29	Czech Republic, Spain, Switzerland	tinea corporis (faciei) tinea capitis	100% similarity with ex-type IHEM 22725 (LR794134), LR794135
T. japonicum	10	Belgium, Czech Republic, France, Japan, Switzerland	tinea corporis	100% similarity with ex-type IHEM 17701 (LR794132)
T. erinacei	23	Czech Republic, France	tinea corporis (faciei) tinea unguium	100% with ex-type CBS 511.73 (LR794136), LT969625- LT969629
T. eriotrephon	1	Iran		100% similarity with JN134090
Trichophyton sp.	4	Iran		MN808768, MF850250

The supernatant was filtered through a Falcon® 40 µm cell strainer (Becton Dickinson) to remove hyphal fragments or clumps. After mild shaking the suspension was diluted to achieve 65- 70% light transmission at wavelength of 530 nm. To attain turbidity equivalent to 0.5 McFarland, corresponding for density of approximately $1 \sim 3 \times 10^3$ CFU/ml, the suspension was diluted in RPMI 1640 medium with the ratio of 1:50. A quantity of 100 µl of twice- serial dilutions of each antifungal was added into

each microplate well along with 100 µl of the inoculum suspension. For each run, dermatophyte- free and antifungal- free controls were used and microplates were incubated at 30°C. The reference strains *T mentagrophytes* TIMM 2789 and *Candida parapsilosis* ATCC 22019 were used as control for every new set of isolates to ensure the accuracy of the antifungals concentration and reproducibility of experiments. The minimal inhibitory concentrations (MICs) for all used antifungals were defined as the lowest concentration that led to complete inhibition of observable growth after 72 h to one week (if needed).

Statistical analysis

The differences between the mean values, relation of MIC levels with species status, geographic location of isolation and type of tinea infection were evaluated statistically using student's *t*- test with SPSS 17.0 software. Statistical significance was set at $p \leq 0.05$.

RESULTS

A total number of 169 isolates was included in the study. Table 1 summarises the molecular identification of isolates and their origin. Most of the isolates originated from Czech Republic (*n* = 124), followed by France (*n* = 19), Iran (*n* = 7), Japan (*n* = 6), Switzerland (*n* = 6), USA (*n* = 3), Belgium (*n* = 3) and Spain (*n* = 1). The ITS- rDNA sequences of all 169 *T. benhamiae* complex strains showed 100% similarity with the ex- type strains or known sequences for which representative genotypes were submitted to GenBank (Tables 1 and Table S1). Based on the ITS- rDNA sequencing, it was found that *T. benhamiae* was the dominant species (*n* = 102), followed by *T- europaeum* (*n* = 29), *T- erinacei* (*n* = 23), *T- japonicum* (*n* = 10), *Trichophyton* sp (*n* = 4) and *T eriotrephon* (*n* = 1). Table 2 lists the geometric mean (GM) MICs, MIC ranges, MIC50 and MIC90 of antifungal agents for the isolates. The GM, and MICs for LUL, TER, EFN, CPO, ITC, GRZ, AMF and TVB across all isolates were as follows, in increasing order: LUL: 0.0004 µg/ ml, TER: 0.027 µg/ml, EFN: 0.044 µg/ml, CPO: 0.18 µg/ml, ITC: 0.44 µg/ml, GRZ: 0.98 µg/ml, AMF: 1.016 µg/ml and TVB: 3.9 µg/ ml. The MIC ranges across all isolates were as follows: LUL: 0.0002- 0.002 µg/ml, TRB: 0.008- 0.125 µg/ml, EFN: 0.008–0 .125 µg/ml, CPO: 0.03– 0.5 µg/ml, ITC: 0.06– 2 µg/ml, GRZ: 0.25– 4 µg/ml, AMF: 0.125– 4 µg/ml and TVB: 1– 16 µg/ml. Furthermore, MIC50/90 for *T benhamiae* isolates (*n* = 102) were as: LUL: 0.0005/0.001 µg/ml, TER: 0.03/0.06 µg/ml, EFN: 0.06/0.06 µg/ml, CPO: 0.25/0.5 µg/ ml, ITC: 0.5/1 µg/ml, GRZ: 1/2 µg/ml, AMF: 1/2 µg/ml and TVB: 4/8 µg/ml. The detailed results are shown in Table 2. No significant differences in the susceptibility patterns of studied species to each antifungal compound were detected. Similarly, no differences in MIC values were found according to geographic origin or clinical manifestation. In Table S1 the results of antifungal susceptibility of all 169 isolates to the applied antifungal agents with respect to the species status and GenBank accession number of isolates were presented.

|DISCUSSION

Zoonotic dermatophytoses due to *T - benhamiae* complex species are highly inflammatory infections with increasing incidence. In recent years, a significant number of studies on these infections have been reported which mainly focused on the clinical aspects of infection, identification, taxonomy or epidemiology.[2- 6]

During the recent years, an alarming increase in the number of difficult- to- treat cases of dermatophytosis indicates that superficial fungal infections remain a global public health concern.[18] One way to overcome such problem is to identify new compounds with antifungal activity. For many years, GRZ has been used as the only systemic antifungal agent for the treatment of dermatophytosis in both humans and animals, while nowadays, there are several antifungals available as alternatives.[19] Due to the increasing incidence of resistance, in vitro antifungal susceptibility tests are frequently used to determine the drug of choice among the available antifungal agents. Epidemiological surveillance of dermatophytosis by using molecular methods for species identification and periodic evaluation of drug resistance / susceptibility patterns in dermatophytes is the only way to prevent the spread of the resistance in the population in its early stage.[20]

The in vitro antifungal susceptibility profile of *T. benhamiae* complex species has been poorly investigated.[21,22] In the present study, for the first time and in accordance to the latest taxonomic recommendations, we provided the antifungal susceptibility profile of many clinical isolates, including those from recently described species such as *T. europaeum*, *T. japonicum* along with *T. benhamiae* s. str., *T. erinacei* and *T. eriotrephon*. For all tested species, LUL, TRB and EFN had lower MICs in comparison with CPO, ITC, GRZ, AMF and TVB. Luliconazole is a newly approved imidazole which has shown a good in vitro activity against major dermatophytes such as *T. rubrum*, *T. mentagrophytes* and *T. interdigitale*.[11,23- 27] As far as we know, this is the first investigation about in vitro activity of LUL against a large collection of clinical isolates from *T. benhamiae* complex.

Similarly to previous studies, LUL showed the lowest MICs (GM: 0.0004 μg/ml) compared to the remaining antifungals tested for all studied species/isolates from different geographic regions. The only exception is the recent study of Zareshahrabadi et al[28] which reported that LUL had low MIC (0.03 μg/ml) for six *T. benhamiae* strains in Shiraz city (Southern Iran). However, ITS- rDNA sequence assessment indicated that isolates collected by Zareshahrabadi et al are phylogenetically distinct from the currently known species in the *T. benhamiae* complex (data not shown). According to molecular and phenotypic data, these isolates cannot be assigned to any currently known species in the *T. benhamiae* complex and probably represent a hitherto undescribed species. According to the excellent activity of LUL against common and dermatophyte new species in the current survey, this antifungal agent has the potential to be considered as the drug of choice in clinical practice.

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Strains and antifungal drug	MIC Range	MIC_{50}/MIC_{90}	Geometric mean	MICs (µg/ml)																	
				16	8	4	2	1	0.5	0.25	0.125	0.06	0.03	0.015	0.008	0.004	0.002	0.001	0.0005	0.0002	0.0001
All strains (*n* = 169)																					
Amorolphine	0.125-4	1/2	1.016			10	38	74	41	5	1										
Ciclopirox	0.03-0.5	0.25/0.5	0.18						43	43	41	41	1								
Greseofulvin	0.25-4	1/2	0.98			3	42	75	45	4											
Terbinafine	0.008-0.125	0.03/0.06	0.027								9	51	47	23	39						
Luliconazole	0.0002-0.002	0.0005/0.001	0.0004														5	31	72	61	
ltraconazole	0.06-2	0.5/1	0.44				3	21	92	49	3	1									
Efinaconazole	0.008-0.125	0.06/0.06	0.044								10	86	59	10	4						
Tavaborole	1-16	4/8	3.9	2	35	92	35	5													
T benhamiae (*n* = 102)																					
Amorolphine	0.125-4	1/2	0.97			5	20	49	25	2	1										
Ciclopirox	0.03-0.5	0.25/0.5	0.17						21	33	24	23	1								
Greseofulvin	0.25-4	1/2	1.06			2	29	47	23	1											
Terbinafine	0.008-0.125	0.03/0.06	0.029								9	28	33	10	22						
Luliconazole	0.0002-0.002	0.0005/0.001	0.0004														2	23	44	33	
Itraconazole	0.06-2	0.5/1	0.45				2	16	55	27	1	1									
Efinaconazole	0.008-0	0.06/0.06	0.042								7	50	35	7	3						
Tavaborole	0.125-4	4/8	4.25	1	27	54	18	2													
T europaeum (*n* = 29)																					

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Amorolphine	0.25-4	1/4	1.07			3	9	7	8	2										
Ciclopirox	0.06-0.5	0.125/0.5	0.13						5	4	9	11								
Greseofulvin	0.25-2	1/2	0.86				7	11	9	2										
Terbinafine	0.008-0.06	0.03/0.06	0.024									10	7	4	8					
Luliconazole	0.0002-0.002	0.0005/0.001	0.0004													1	4	14	10	
Itraconazole	0.125-1	0.5/1	0.39					3	15	9	2									
Efinaconazole	0.008-0.125	0.06/0.06	0.045								2	15	11		1					
Tavaborole	1-16	4/8	4.096	1	4	21	1	2												
T erinacei (*n* = 23)																				
Amorolphine	0.25-2	1/2	1.062				6	14	2	1										
Ciclopirox	0.06-0.5	0.25/0.5	0.26						11	6	2	4								
Greseofulvin	0.5-4	1/2	0.97			1	4	11	7											

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Strains and antifungal drug	MIC Range	MIC_{50}/MIC_{90}	Geometric mean	MICs (µg/ml)																
				16	8	4	2	1	0.5	0.25	0.125	0.06	0.03	0.015	0.008	0.004	0.002	0.001	0.0005	0.0002	0.0001
Terbinafine	0.008-0.06	0.03/0.06	0.030									11	4	5	3						
Luliconazole	0.0002-0.002	0.0005/0.001	0.0004														1	3	11	8	
Itraconazole	0.25-2	0.5/1	0.43				1	2	11	9											
Efinaconazole	0.015-0.06	0.03/0.06	0.038									11	9	3							
Tavaborole	1-8	2/8	2.95		3	8	11	1													
T japonicum (n = 10)																					
Amorolphine	0.5-4	0.5/2	0.87			1	2	1	6												
Ciclopirox	0.06-0.5	0.125/0.125	0.11						1		6	3									
Greseofulvin	0.25-2	0.5/2	0.7				2	2	5	1											
Terbinafine	0.008-0.06	0.008/0.03	0.014									1	2	2	5						
Luliconazole	0.0002-0.001	0.0002/0.0005	0.0003															1	3	6	
ltraconazole	0.25-0.5	0.5/0.5	0.4						7	3											
Efinaconazole	0.03-0.125	0.06/0.06	0.06								1	8	1								
Tavaborole	2-8	4/4	4		1	8	1														
Trichophyton sp (n = 4)																					
Amorolphine	1-4	-	-			1	1	2													
Ciclopirox	0.5	-	-						4												
Greseofulvin	0.5-1	-	-					3	1												

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Terbinafine	0.015-0.06	-	-						1	1	2			
Luliconazole	0.0002-0.002	-	-											4
Itraconazole	0.5	-	-				4							
Efinaconazole	0.03-0.06	-	-						2	2				
Tavaborole	2	-	-		4									
T eriotrephon (*n* = 1)														
Amorolphine	1	-	-			*								
Ciclopirox	0.5	-	-				*							
Greseofulvin	1	-	-			*								
Terbinafine												*		
Luliconazole	0.002	-	-										*	
Itraconazole	0.25	-	-					*						
Efinaconazole	0.03	-	-							*				
Tavaborole	4	-	-	*										

Efinaconazole is another member of azole antifungal group that was approved in 2014, especially for the treatment of onychomycosis in human.[29] A remarkable feature of EFN is its ability to penetrate the nail bed to a greater extent than other topical antifungal drugs.[30] In previous studies, it was shown that EFN has good in vitro activity against some anthropophilic, zoophilic and geophilic dermatophytes.[11,25,31] The good activity of EFN (GM: 0.044 µg/ ml) against members of the *T benhamiae* complex in current investigation corroborated this fact. Terbinafine has widely been shown to be very effective in the treatment of human and animal dermatophytosis and has a high therapeutic rating.[32] The results of the present study showed a low MIC of TRB (GM: 0.027 µg/ml) against all tested dermatophytes which was comparable with findings of Ansari et al,[21] who showed that TRB (GM: 0.025 µg/ml) had the lowest MIC against Iranian *Trichophyton* sp isolates. Despite good in vitro activity of TRB against several anthropophilic and zoophilic species,[21,25,28,33-38] several recent surveys reported the growing incidence of TRB resistance among dermatophytes,.[8,11,39] However, no case of such in vitro resistance was found among our *T benhamiae* complex isolates. Ciclopirox olamine is a derivative of hydroxypyridone that is marketed in many forms and used for treatment of fungal skin, nail and scalp infections in humans.[40] Compared to LUL, EFN and TRB, CPO has poorly been the matter of antifungal evaluation and we found no data concerning in vitro efficacy of CPO agent against members of *T benhamiae* complex. However, CPO has shown a considerable activity against other dermatophytes[8,25,26] and in the current assessment, it also showed a good in vitro activity (GM: 0.18 µg/ml) against all studied species. AMF is a morpholine derivative with fungistatic and antifungal activity against dermatophytes and some pathogenic moulds and yeasts.[41] In previous studies, AMF showed higher activity to dermatophytes with GM ranged 0.0245– 0.24 µg/ml,[20,26] while, in our study it had low activity (GM: 1.016 µg/ml) against *T. benhamiae* complex. Itraconazole is one of the anti-dermatophyte drugs that is effective against most forms of dermatophytosis in humans and companion animals.[19] Although, in the present study, ITC had low MIC (GM: 0.44 µg/ml) against *T. benhamiae* complex isolates, it was higher than those reported by Ansari et al[21] (GM: 0.05 µg/ml). Historically, griseofulvin has been the first drug of choice for systemic treatment of dermatophytosis.[19] Similarly to Ansari et al,[21] GRZ showed low activity (GM: 0.98 µg/ml) against *T. benhamiae* complex isolates from the present study. Finally, TVB is a new FDA- approved antifungal agent with broad- spectrum antifungal activity against yeasts, non- dermatophyte moulds and dermatophytes involved in human onychomycosis.[42] Similarly to CPO, its potent activity against dermatophytes has poorly been investigated. In the study of Coronado et al,[43] the MIC values of TVB for 19 strains of *T. rubrum* and *T. mentagrophytes* ranged from 0.25 to 16 µg/ml. Recently, Abastabar et al,[44] also reported low in vitro activity of TVB (MIC = 4– 16 µg/ml) against *T. interdigitale* (*n* = 6), *T rubrum* (*n* = 8) and *T. tonsurans* (*n* = 1) isolates from human onychomycosis. Among the antifungal agents examined in this study, TVB had the highest MICs (GM: 3.9 µg/ml). However we obtained MIC values about twice lower than those reported by Abastabar et al Generally, little information is available on the susceptibility of isolates

included in the present study to antifungal agents and consequently, it is difficult to explain observed differences in antifungal susceptibility patterns compared to previous studied.

Some recent studies about *T. violaceum*,[34] *T. schoenleinii*,[35] *M. canis*[45] and *T. rubrum*[46] isolates obtained from different countries, including Iran, Turkey, China and France, showed that MIC values are independent of their geographic region of isolation and type of infection. On the other hand, there have been many reports indicating that some clinical strains/lineages of *T. mentagrophytes* and *T. rubrum* show elevated TRB MICs and harbour mutations in the squalene epoxidase (*SQLE*) gene depending on their geographic origin. Terbinafine- resistant isolates of *T. mentagrophytes*, also known as *T. mentagrophytes* genotype VIII or *T. indotineae*, were initially predominantly reported from India, but are now reported from other parts of the world.[8,10- 12,36,47] In the present study, changes in the taxonomic status and differences in geographic origin have not been statistically associated with difference in the mean values of tested antifungals. No evidence of drug resistance, particularly in vitro resistance to TRB, was found.

CONCLUSIONS

The present investigation provided valuable insights on the validity of sequence based method for identification of closely related species in the *T. benhamiae* species complex. Future studies with standardised methods are needed to correlates the MIC values of tested antifungals against *T. benhamiae* complex isolates with clinical consequence and allow clinicians to adopt different antifungal treatment options with a high probability of successful outcomes.

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CONFLICT OF INTEREST

All authors declare no conflicts of interest relevant to this article.

AUTHOR CONTRIBUTIONS

Forough Shamsizadeh: Data curation (equal); Investigation (equal); Methodology (equal); Writing- original draft (equal). **Saham Ansari:** Data curation (equal); Writing- original draft (equal). **Ali Zarei Mahmoudabadi:** Data curation (equal); Writing- original draft (equal). **Vit Hubka:** Data curation (equal); Writing- review & editing (equal). **Adéla Čmoková:** Data curation (equal); Writing- review &

editing (equal). **Jacques Guillot:** Data curation (equal); Writing- review & editing (equal). **Abdollah Rafiei:** Data curation (equal). **Kamiar Zomorodian:** Data curation (equal). **Sadegh Nouripour-Sisakht:** Data curation (equal). **Kambiz Diba:** Data curation (equal). **Tooba Mohammadi:** Data curation (equal). **Hossein Zarrinfar:** Data curation (equal). **Ali Rezaei- Matehkolaei:** Conceptualization (equal); Data curation (equal); Project administration (equal); Supervision (equal); Writing- original draft (equal).

AUTHOR CONTRIBUTIONS

AR- M conceived the idea; FS performed the experiments; all authors collected the samples and data; AR- M, FS, SA and AZ- M drafted the manuscript, VH, AC and JG modified and extended the draft.

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Table S1. MIC values (µg/mL) of antifungals against used in the study against each *Trichophyton benhamiae* complex isolate

Species	Sample ID	Accession Number	Antifungals/MIC (µg/mL)							
			Amorolphine	Ciclopirox	Griseofulvin	Terbinafine	Luliconazole	Itraconazole	Efinaconazole	Tavaborole
T. benhamiae	D 620.18	LR794129	1	0.25	1	0.0625	0.000937	0.5	0.0625	8
	P 358.18	LR794129	1	0.25	1	0.0312	0.000468	1	0.0312	8
	CLIS6616.18	LR794129	2	0.25	2	0.0625	0.000937	0.5	0.0625	4
	KDVB121.18	LR794129	0.5	0.125	0.5	0.0156	0.000234	0.5	0.00781	4
	ME 241.18	LR794129	1	0.125	2	0.0156	0.000234	0.25	0.00781	8
	ME 438.18	LR794129	0.5	0.125	0.5	0.00781	0.000234	0.5	0.0156	8
	CLIS5383.18	LR794129	0.5	0.0625	2	0.00781	0.000234	0.5	0.00781	4
	D 904.18	LR794129	0.25	0.125	1	0.00781	0.000234	0.25	0.0156	4
	CLIS 4424	LR794129	0.25	0.0625	2	0.00781	0.000468	0.25	0.0625	2
	KDVB88.19	LR794129	0.5	0.125	0.5	0.00781	0.000468	0.25	0.0156	8
	CLIS 3606	LR794129	1	0.0625	0.5	0.0625	0.000468	0.25	0.0625	4
	SK 213	LR794129	1	0.0625	2	0.0625	0.000234	0.5	0.0625	4
	P 270.18	LR794129	1	0.25	1	0.00781	0.000234	0.5	0.0625	4
	D 900.18	LR794129	0.5	0.125	1	0.0156	0.000234	0.25	0.0312	8
	KDVB 90.19	LR794129	1	0.125	1	0.0156	0.000468	0.25	0.0625	4
	ME 378.18	LR794129	0.5	0.125	0.5	0.0156	0.000234	0.5	0.125	2
	KDVB 55.18	LR794129	1	0.125	1	0.0312	0.000468	0.5	0.0625	8
	ME 566.18	LR794129	1	0.25	1	0.0312	0.000234	0.5	0.0625	4
	CLIS 6870.18	LR794129	1	0.0625	1	0.0312	0.000468	0.25	0.0625	4
	CLIS 4948.18	LR794129	0.5	0.25	0.5	0.00781	0.000234	0.5	0.0625	4
	CLIS 233	LR794129	1	0.0625	1	0.0625	0.000468	0.25	0.0312	4

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	SK 3649	LR794129	0.5	0.25	0.5	0.0312	0.000468	0.25	0.0156	4
	CLIS 8142	LR794129	1	0.25	1	0.0312	0.000468	0.25	0.0156	4
	CLIS 5129	LR794129	1	0.0625	2	0.0625	0.000468	0.5	0.0312	8
	SK 2345.17	LR794129	1	0.0625	4	0.125	0.000234	0.5	0.0625	8
	SK 53	LR794129	2	0.5	2	0.0312	0.000937	0.25	0.0312	16
	ME 86	LR794129	1	0.25	1	0.0312	0.000468	0.5	0.0625	4
	SK 2209.18	LR794129	0.5	0.25	1	0.125	0.000468	1	0.0312	4
	CLIS 5776.18	LR794129	0.5	0.25	0.5	0.0312	0.000468	0.5	0.0312	4
	CLIS 3640	LR794129	1	0.125	4	0.0156	0.000234	0.125	0.0156	4
	CLIS 3048	LR794129	2	0.5	2	0.0312	0.000234	0.25	0.0312	4
	KDVB 43.8	LR794129	1	0.0625	2	0.0625	0.000234	0.5	0.125	2
T. benhamiae	SK 2445.18	LR794129	2	0.5	1	0.0312	0.000468	0.25	0.0625	8
	KDVB 23.19	LR794129	0.5	0.25	0.5	0.0312	0.000468	2	0.0312	8
	D 237	LR794129	0.5	0.5	2	0.0625	0.000468	0.5	0.0625	2
	SK 30	LR794129	1	0.25	2	0.125	0.000937	0.5	0.0625	4
	KDVB 129.18	LR794129	1	0.25	1	0.125	0.000937	0.5	0.0625	4
	CLIS 9763.18	LR794129	1	0.25	1	0.0312	0.000468	1	0.0625	2
	SK 2339.17	LR794129	2	0.5	2	0.0312	0.000468	0.5	0.0625	8
	SK 3660.17	LR794129	1	0.5	2	0.0625	0.000937	1	0.0312	4
	SK 2075.17	LR794129	2	0.25	2	0.0625	0.000937	0.5	0.0625	4
	SK 4402.18	LR794129	0.5	0.125	2	0.125	0.000468	0.5	0.0625	4
	SK 4121	LR794129	2	0.5	2	0.0625	0.000468	0.5	0.0625	4
	CLIS 4348	LR794129	1	0.0625	1	0.0625	0.000468	1	0.0625	2
	KDVB 9.19	LR794129	0.5	0.5	0.5	0.0312	0.000234	1	0.0625	4
	KDVB 56.18	LR794129	0.5	0.125	0.5	0.0156	0.000468	0.5	0.0312	4

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	KDVB 56.19	LR794129	0.5	0.125	0.5	0.0156	0.000468	0.5	0.0312	4
	D 296	LR794129	1	0.25	1	0.0312	0.000937	0.25	0.0312	4
	SK 2905.18	LR794129	1	0.25	1	0.0312	0.000937	0.5	0.0625	8
	ME 439.18	LR794129	0.5	0.25	1	0.0625	0.000468	0.5	0.0312	4
	KDVB 3.18	LR794129	4	0.25	2	0.0625	0.000937	0.25	0.0312	8
	SK 712.17	LR794129	1	0.25	1	0.125	0.000937	1	0.0625	8
	P 268.18	LR794129	1	0.0625	1	0.0312	0.000468	0.25	0.0156	4
	CLIS 260	LR794129	0.5	0.25	1	0.0312	0.000468	0.5	0.0312	4
	CLIS 1713	LR794129	4	0.125	0.5	0.125	0.000937	0.25	0.0312	4
	CLIS 7074	LR794129	1	0.25	1	0.0625	0.000937	0.5	0.0312	4
	CLIS 8917	LR794129	1	0.25	1	0.0312	0.000468	2	0.0625	4
	CLIS 5067	LR794129	4	0.5	1	0.0312	0.000937	1	0.0625	4
	CLIS 5367	LR794129	1	0.125	0.5	0.0312	0.000234	0.5	0.0312	4
	CLIS 6279.18	LR794129	1	0.25	1	0.0625	0.000937	0.5	0.125	8
	CLIS 6219.18	LR794129	2	0.125	1	0.0625	0.000937	0.5	0.125	8
	KDVB 29.19	LR794129	0.5	0.25	2	0.0625	0.00187	0.5	0.0625	4
	SK 51	LR794129	2	0.125	2	0.125	0.000937	1	0.125	4
	SK 2906.18	LR794129	1	0.25	2	0.0625	0.000937	0.5	0.0625	4
	D 187	LR794129	4	0.25	2	0.125	0.000468	0.5	0.0312	8
	D 943.18	LR794129	2	0.5	2	0.0625	0.000468	0.5	0.0625	8
	P 206.18	LR794129	1	0.5	2	0.0625	0.000937	1	0.125	8
	P 208	LR794129	1	0.5	1	0.0625	0.000468	0.25	0.0625	4
	D 642.18	LR794129	0.125	0.5	2	0.0625	0.000937	1	0.125	4
T. benhamiae	SK 1948	LR794129	1	0.25	1	0.0312	0.000468	0.5	0.0312	8
	IHEM 25077	LR794129	1	0.125	1	0.00781	0.000468	0.5	0.0625	4
	IHEM 3287	LR794129	2	0.0625	1	0.00781	0.000234	0.25	0.0312	4

IHEM 25742	LR794129	1	0.125	1	0.00781	0.000234	0.25	0.0625	4
USA 3366	LR794129	0.5	0.0625	1	0.00781	0.000234	0.5	0.0625	4
USA 3350	LR794129	2	0.0625	0.5	0.0625	0.000234	0.5	0.0625	8
NUBS 13002	LR794129	0.5	0.0625	0.5	0.0312	0.000234	0.25	0.0625	4
IHEM 24744	LR794129	1	0.25	0.5	0.00781	0.000468	1	0.0625	4
CCF 4852	LR794129	0.5	0.125	0.5	0.0312	0.000937	0.5	0.0625	4
DMF 813	LR794129	0.5	0.0312	1	0.00781	0.000234	0.5	0.0625	4
DMF 1869	LR794129	2	0.25	1	0.00781	0.000234	0.5	0.0312	8
CCF 4918	LR794129	1	0.5	1	0.0625	0.000468	0.5	0.0312	4
ME 67	LR794129	0.5	0.0625	1	0.00781	0.000234	1	0.0312	4
CCF 4847	LR794129	1	0.125	0.5	0.00781	0.000468	0.5	0.0312	2
CCF 4798	LR794129	2	0.125	0.5	0.0156	0.000234	1	0.0625	8
SK 3941.13	LR794129	1	0.0625	2	0.00781	0.000234	0.5	0.0625	8
CCF 4851	LR794129	1	0.0625	0.25	0.0156	0.000234	0.5	0.0625	2
SK 818	LR794129	1	0.0625	1	0.00781	0.000234	0.25	0.0312	4
CCF 4850	LR794129	1	0.25	1	0.00781	0.000468	0.25	0.0625	2
CCF 4849	LR794129	4	0.0625	0.5	0.0312	0.000234	0.25	0.0625	2
CCF 4854	LR794129	1	0.125	0.5	0.0312	0.000234	0.5	0.0312	2
CCF 4855	LK054798	1	0.125	1	0.00781	0.000234	0.5	0.0625	2
DMF 1072	LR794129	2	0.0625	1	0.00781	0.000234	0.5	0.0625	1
SK 855.13	LR794129	0.5	0.0625	1	0.00781	0.000468	0.25	0.0312	8
CLIS 3401	LR794129	2	0.25	1	0.0312	0.000937	0.5	0.0312	2
DMF 317.12	LR794129	1	0.0625	0.5	0.0312	0.000468	1	0.0312	4
T 2122	LR794129	2	0.5	2	0.0625	0.000468	0.5	0.0312	2
W 1159	LR794129	2	0.5	1	0.0625	0.000468	1	0.0625	2
W 1350	LR794129	1	0.5	2	0.0312	0.00187	0.5	0.0625	2
W 1360	LR794129	1	0.5	2	0.0312	0.000468	0.0625	0.0625	1
X 491	LR794129	1	0.5	2	0.0625	0.000937	0.25	0.0312	8
UR 1	MN808769	2	0.5	1	0.0312	0.000468	0.5	0.0312	2

	UR 59	MN808770	2	0.5	1	0.0312	0.000468	0.5	0.0312	2
Trichophyton sp	MJN - 81	MF850250	1	0.5	1	0.0625	0.000234	0.5	0.0312	2
	MJN - 91	MF850250	2	0.5	1	0.0156	0.000234	0.5	0.0312	2
	S - 394	MF850250	4	0.5	0.5	0.0156	0.000234	0.5	0.0625	2
	SH - 1	MN808768	1	0.5	1	0.0312	0.000234	0.5	0.0625	2
T. europaeum	CLIS 4864	LC794134	0.5	0.0625	1	0.0625	0.000468	0.5	0.0312	4
	CLIS 9615	LC794134	0.5	0.0625	1	0.0312	0.000468	0.5	0.0312	1
	P 184.18	LC794134	1	0.125	1	0.00781	0.000234	0.25	0.00781	8
	SK 4193.17	LC794134	2	0.25	1	0.0312	0.000468	0.5	0.0312	4
	CLIS 10039	LC794134	1	0.125	1	0.0625	0.000937	1	0.0625	4
	CLIS10188	LC794134	0.5	0.25	2	0.0625	0.000468	0.5	0.0625	16
	CLIS10202.18	LC794134	2	0.5	2	0.0625	0.000937	0.5	0.0625	8
	SK 2958	LC794134	2	0.5	2	0.0625	0.000468	0.5	0.0625	4
	CLIS 9483	LC794134	0.5	0.0625	2	0.0625	0.000234	0.5	0.0625	4
	SK 1151.17	LC794134	0.25	0.25	0.5	0.00781	0.000468	1	0.0625	4
	KDVB 86.18	LC794134	1	0.0625	0.25	0.0312	0.000468	0.125	0.0625	4
	SK 57	LC794134	2	0.5	1	0.0625	0.000937	0.5	0.0312	4
	CLIS 421	LC794134	2	0.5	2	0.0625	0.00187	1	0.0312	2
	CLIS 2488.18	LC794134	2	0.25	0.5	0.0625	0.000937	0.5	0.0625	4
	ME 192.12	LC794134	1	0.125	0.5	0.00781	0.000234	0.25	0.0625	4
	IHEM 25139	LC794135	0.5	0.125	0.5	0.0156	0.000468	0.5	0.125	4
	IHEM 20161	LC794134	4	0.0625	1	0.0312	0.000234	0.25	0.0625	4
	IHEM 25164	LC794134	4	0.0625	0.5	0.0625	0.000234	0.25	0.125	4
	IHEM 22727	LC794134	0.5	0.125	0.25	0.0312	0.000468	0.5	0.0312	8

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	IHEM 25062	LC794134	2	0.125	0.5	0.00781	0.000234	0.25	0.0312	4
	DMF 2994	LC794134	0.25	0.125	2	0.0312	0.000468	0.5	0.0312	4
	ME 962.11	LC794134	0.5	0.125	1	0.00781	0.000234	0.5	0.0625	4
	CCF 4848	LC794134	1	0.5	0.5	0.00781	0.000468	0.25	0.0312	4
	CCF 4917	LC794134	1	0.0625	0.5	0.0156	0.000468	0.125	0.0312	4
	DMF 493	LC794134	4	0.125	2	0.0312	0.000468	0.5	0.0625	4
	CCF 4853	LC794134	1	0.0625	0.5	0.0156	0.000468	0.25	0.0625	8
	SK 3055	LC794134	0.5	0.0625	1	0.00781	0.000234	0.25	0.0625	4
	ME 1187.11	LC794134	2	0.0625	1	0.00781	0.000234	0.5	0.0625	1
	DMF 3016.12	LC794134	2	0.0625	1	0.0156	0.000234	0.25	0.0312	4
	ME 1495	LT969625	1	0.25	4	0.0312	0.000468	0.5	0.0625	8
	CLIS 1516	LR794136	1	0.5	2	0.0625	0.000937	1	0.0312	4
	CCF 5727	LT969629	2	0.5	0.5	0.0625	0.000468	0.25	0.0312	1
	CCF 5930	LT969629	0.5	0.25	2	0.0625	0.000234	0.25	0.0312	8
	CCF 5931	LT969627	1	0.25	1	0.0156	0.000234	0.5	0.0625	8
	CCF 5775	LT969625	1	0.125	1	0.0156	0.000234	0.5	0.0625	4
	CCF 5209	LT969629	1	0.0625	2	0.0156	0.000234	0.5	0.0312	4
	CCF 5864	LT969626	0.25	0.0625	0.5	0.00781	0.000468	1	0.0156	4
	CCF 3946	LT969625	1	0.0625	1	0.00781	0.000937	0.5	0.0625	4
	CCF 5929	LT969625	1	0.0625	0.5	0.00781	0.000234	0.25	0.0625	4
T. erinacei	W 1129	LT969629	1	0.5	0.5	0.0625	0.000468	0.25	0.0625	2
	W 1204	LT969629	0.5	0.5	1	0.0312	0.000937	0.5	0.0625	2
	W 1410	LT969629	1	0.25	1	0.0312	0.000468	0.5	0.0156	2
	W 1595	LT969629	2	0.5	0.5	0.0312	0.000234	0.25	0.0312	2
	W 1173	LT969629	1	0.125	1	0.0625	0.00187	0.5	0.0156	2
	W 1349	LT969629	1	0.5	1	0.0625	0.000234	2	0.0625	2
	T 11339	LT969629	2	0.25	1	0.0156	0.000234	0.5	0.0625	2
	W 1543	LT969629	2	0.25	2	0.0625	0.000468	0.25	0.0312	4
	W 1477.2	LT969629	1	0.5	1	0.0625	0.000468	0.25	0.0312	2
	X 69	LT969629	1	0.5	0.5	0.0625	0.000468	0.25	0.0312	2

	W 1505.1	MH860764	2	0.5	1	0.0156	0.000468	0.25	0.0625	2
	w 1545	LT969629	2	0.5	1	0.0625	0.000468	0.5	0.0625	2
	W 1542.1	LT969629	1	0.5	0.5	0.0625	0.000468	0.5	0.0312	4
T. japonicum	SK 3467.18	LR794132	2	0.5	1	0.0625	0.000468	0.5	0.0625	4
	VUT 00001	LR794132	0.5	0.0625	0.5	0.00781	0.000234	0.25	0.0625	4
	JPN 7	LR794132	0.5	0.125	0.5	0.00781	0.000234	0.5	0.125	4
	JPN 10	LR794132	2	0.125	2	0.0156	0.000234	0.5	0.0625	8
	NUBS 120001	LR794132	0.5	0.125	0.5	0.0156	0.000468	0.5	0.0625	4
	VUT 97010	LR794132	1	0.125	0.25	0.00781	0.000234	0.5	0.0625	4
	IHEM 17701	LR794132	0.5	0.125	0.5	0.00781	0.000468	0.5	0.0625	4
	IHEM 25063	LR794132	0.5	0.125	0.5	0.00781	0.000234	0.25	0.0625	4
	IHEM 4030	LR794132	4	0.0625	1	0.0312	0.000234	0.25	0.0312	4
	CCF 4799	LR794132	0.5	0.0625	2	0.0312	0.000937	0.5	0.0625	2
T. eriotrephon	M-12	JN134090	1	0.5	1	0.00781	0.00187	0.25	0.0312	4

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14) **Additional Terms for Specific Products and Services.** If a User is making one of the uses described in this Section 14, the additional terms and conditions apply:

a) ***Print Uses of Academic Course Content and Materials (photocopies for academic coursepacks or classroom handouts).*** For photocopies for academic coursepacks or classroom handouts the following additional terms apply:

i) The copies and anthologies created under this License may be made and assembled by faculty members individually or at their request by on-campus bookstores or copy centers, or by off-campus copy shops and other similar entities.

ii) No License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied) (ii) permit "publishing ventures" where any particular anthology would be systematically marketed at multiple institutions.

iii) Subject to any Publisher Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the academic pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to no more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular anthology, whether photocopied or electronic, at more than one institution of learning;

E) in the case of a photocopy permission, no materials may be entered into electronic memory by User except in order to produce an identical copy of a Work before or during the academic term (or analogous period) as to which any particular permission is granted. In the event that User shall choose to retain materials that are the subject of a photocopy permission in electronic memory for purposes of producing identical copies more than one day after such retention (but still within the scope of any permission granted), User must notify CCC of such fact in the applicable permission request and such retention shall constitute one copy actually sold for purposes of calculating permission fees due; and

F) any permission granted shall expire at the end of the class. No permission granted shall in any way include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied).

iv) Books and Records; Right to Audit. As to each permission granted under the academic pay-per-use Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any photocopies sold or by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date

such amoun was originally due. The provisions of this paragraph shall survive the termination of this License for any reason.

b) ***Digital Pay-Per-Uses of Academic Course Content and Materials (e-coursepacks, electronic reserves, learning management systems, academic institution intranets).*** For uses in e-coursepacks, posts in electronic reserves, posts in learning management systems, or posts on academic institution intranets, the following additional terms apply:

i) The pay-per-uses subject to this Section 14(b) include:

A) **Posting e-reserves, course management systems, e-coursepacks for text-based content,** which grants authorizations to import requested material in electronic format, and allows electronic access to this material to members of a designated college or university class, under the direction of an instructor designated by the college or university, accessible only under appropriate electronic controls (e.g., password);

B) **Posting e-reserves, course management systems, e-coursepacks for material consisting of photographs or other still images not embedded in text,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorization: to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above, including any necessary resizing, reformatting or modification of the resolution of such requested material (provided that such modification does not alter the underlying editorial content or meaning of the requested material, and provided that the resulting modified content is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms), but not including any other form of manipulation, alteration or editing of the requested material;

C) **Posting e-reserves, course management systems, e-coursepacks or other academic distribution for audiovisual content,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorizations: (i) to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above; (ii) to display and perform the requested material to such members of such class in the physical classroom or remotely by means of streaming media or other video formats; and (iii) to "clip" or reformat the requested material for purposes of time or content management or ease of delivery, provided that such "clipping" or reformatting does not alter the underlying editorial content or meaning of the requested material and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms. Unless expressly set forth in the relevant Order Conformation, the License does not authorize any other form of manipulation, alteration or editing of the requested material.

ii) Unless expressly set forth in the relevant Order Confirmation, no License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied or, in the case of Works subject to Sections 14(b)(1)(B) or (C) above, as described in such Sections) (ii) permit "publishing ventures" where any particular course materials would be systematically marketed at multiple institutions.

iii) Subject to any further limitations determined in the Rightsholder Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the electronic course content pay-per-use service is limited as follows:

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B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular materials, whether photocopied or electronic, at more than one institution of learning;

E) electronic access to material which is the subject of an electronic-use permission must be limited by means of electronic password, student identification or other control permitting access solely to students and instructors in the class;

F) User must ensure (through use of an electronic cover page or other appropriate means) that any person, upon gaining electronic access to the material, which is the subject of a permission, shall see:

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- a sta ement to the effect that such copy was made pursuant to permission,
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v) Books and Records; Right to Audit. As to each permission granted under the electronic course content Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any electronic copies used by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this license for any reason.

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15) **Miscellaneous.**

a) User acknowledges that CCC may, from time to time, make changes or additions to the Service or to the Terms, and that Rightsholder may make changes or additions to the Rightsholder Terms. Such updated Terms will replace the prior terms and conditions in the order workflow and shall be effective as to any subsequent Licenses but shall not apply to Licenses already granted and paid for under a prior set of terms.

b) Use of User-related information collected through the Service is governed by CCC's privacy policy, available online at www.copyright.com/about/privacy-policy/.

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e) The License described in the Order Confirmation shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such License shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.

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