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MINIREVIEW

# Genetic advances in dermatophytes

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## Abstract

Millions of superficial fungal infections are annually observed in humans and animals. The majority of these mycoses are caused by dermatophytes, a specialized group of filamentous fungi that exclusively infect keratinized host structures. Despite the high prevalence of the disease, dermatophytosis, little is known about the pathogenicity mechanisms of these microorganisms. This drawback may be related to the fact that dermatophytes have been investigated poorly at the molecular level. In contrast to many other pathogenic fungi, they grow comparatively slowly under *in vitro* conditions, and in the last decades, only a limited number of molecular tools have been established for their manipulation. In recent years, however, major promising approaches were undertaken to improve genetic analyses in dermatophytes. These strategies include efficient systems for targeted gene inactivation and gene silencing, and broad transcriptional profiling techniques, which have even been applied in sophisticated infection models. As a fundamental prerequisite for future genetic analyses, full genome sequences of seven different dermatophyte species have become available recently. Therefore, it appeared timely to review the available molecular tools and methodologies in dermatophyte research, which may provide future insights into the virulence of these clinically important pathogens.

## Introduction

Genetic approaches have allowed fundamental insights into almost all areas of microbial pathogenesis research. Yet, today, such methodologies have only rarely been established in dermatophytes, in contrast to other clinically important fungal pathogens, for example *Candida albicans*, *Aspergillus fumigatus* or *Cryptococcus neoformans*. Consequently, little is known about the pathogenicity of dermatophytes at the molecular level. Dermatophytes constitute a group of highly specialized filamentous fungi that share the peculiar ability to digest and grow on keratinized host structures such as skin stratum corneum, hair and nails (Fig. 1) (Ajello, 1974). Keratin utilization by these microorganisms as the sole carbon and nitrogen source has been linked to extracellular proteolysis, and a large number of secreted proteases were identified in different dermatophyte species (reviewed in Monod, 2008). Despite these major efforts, however, the role of individual proteases during infection remains almost elusive. Moreover, dermatophyte pathogenicity likely tends to be more complex and involves fungal mechanisms that still have to be identified. At the same time, it appears to be of particular note that the adaptation of dermatophytes to specific host niches is associated with variable clinical signs, i.e. chronic vs. inflammatory disease, suggesting distinct, almost unknown pathophysiological reactions. Therefore, studies on dermatophyte pathogenicity focus not only on fungal attributes but also on host immune response mechanisms (Vermout *et al.*, 2008; Brasch, 2009).

Comprehensive up-to-date review articles covering dermatophyte epidemiology and clinical importance as well as genetic approaches in taxonomy and diagnosis are already available (Binstock, 2007; Abdel-Rahman, 2008; Gräser *et al.*, 2008; Kanbe, 2008; Seebacher *et al.*, 2008; Ameen, 2010). These topics will not be a part of the present overview. Nevertheless, some basic information on species diversity and medical impact will be provided in order to better convey the recent achievements in molecular genetic research in this fascinating group of microorganisms.

## Dermatophytes: clinical importance and taxonomy

Dermatophytoses belong to the most common infectious diseases in humans, affecting 10–20% of the population

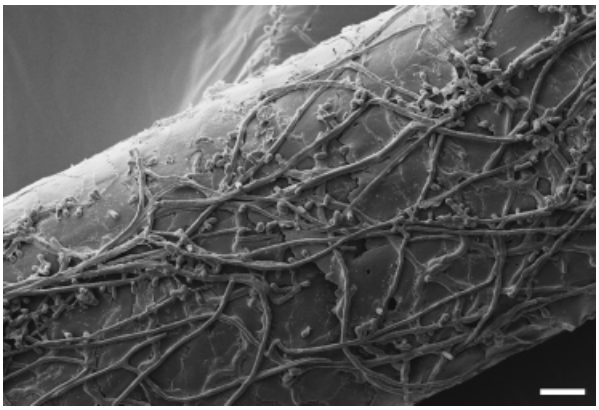

**Fig. 1.** Scanning electron micrograph of the dermatophyte *Arthroderma benhamiae* colonizing human hair. Sterilized hair was infected with *A. benhamiae* microconidia and incubated for 14 days at 30 °C. Scale bar = 10 μm.

worldwide. These infections are usually not life threatening, but occur even in immunocompetent hosts, and in many cases, are long lasting, recurrent and difficult to cure (Borgers *et al.*, 2005). Depending on their predominant natural reservoir, dermatophyte species are classified into three groups: anthropophilic, zoophilic and geophilic (Weitzman & Summerbell, 1995). The natural hosts of anthropophilic and zoophilic species are humans and animals, respectively, whereas geophilic dermatophytes are soil saprophytes. Symptoms of dermatophytosis can vary from chronic to highly inflammatory, depending on the causative agent and the body location affected. The given disease is described with the word 'tinea,' followed by a term referring to the infected body site, for example tinea pedis (feet), tinea capitis (scalp or head), tinea corporis (body or trunk) and tinea unguium (nails, also called onychomycosis) (Degreef, 2008). Major prominent anthropophilic species, for example, *Trichophyton rubrum*, *Trichophyton interdigitale* and *Trichophyton tonsurans*, are mostly associated with more chronic, less inflammatory infections. In contrast, zoophilic species, for example, *Microsporum canis*, *Arthroderma benhamiae*, *Arthroderma vanbreuseghemii*, *Trichophyton erinacei* and *Trichophyton verrucosum* as well as geophilic dermatophytes such as *Microsporum gypseum* often induce highly inflamed lesions in humans.

Dermatophytes are ascomycete fungi. The anamorphs (asexual forms) are classified into three genera: *Trichophyton*, *Microsporum* and *Epidermophyton*. Teleomorphs (sexual forms) belong to the *Arthroderma* genus in the Ascomycotina subphylum. Dermatophytes are heterothallic (mating types are designated as either '+' or '−'); however, in many zoophilic and anthropophilic species, sexual reproduction has not been observed. Recent progress in molecular taxonomy and insights into mating revealed that *Trichophyton mentagrophytes* was a complex of anthropophilic and zoophilic species that produce different teleomorphs, leading to a current confusion in species denomination. For example, *A. benhamiae* is the teleomorph obtained by mating isolates from rodents (Ajello & Cheng, 1967), whereas *A. vanbreuseghemii* is the teleomorph from strains isolated from humans and certain rodents (Takashio, 1979). Both zoophilic species *A. benhamiae* and *A. vanbreuseghemii* cause highly inflammatory tinea capitis, tinea corporis and tinea faciei. They are designated *T. mentagrophytes* and *T. mentagrophytes* var. *asteroides* in many textbooks and publications. The anthropophilic strains of the *T. mentagrophytes* species complex produce noninflammatory tinea pedis and tinea unguium. Sexual reproduction has not been observed and the fungus is still called by the anamorph name *T. interdigitale* (or *T. mentagrophytes* var. *interdigitale*) (Symoens *et al.*, 2011). Therefore, the formerly widely used species description, *T. mentagrophytes*, should nowadays only be used for isolates referring to the reference strain designated as a neotype (Gräser *et al.*, 1999). This hint appears to be noteworthy, because many of the genetic studies in dermatophytes were performed using species of the *T. mentagrophytes* complex, i.e. *A. benhamiae* and *A. vanbreuseghemii*. However, in the case of the latter species, the name *T. mentagrophytes* was used (e.g. Yamada *et al.*, 2005, 2008, 2009a, b; Alshahni *et al.*, 2011).

## Broad-scale gene discovery in dermatophytes

### Transcriptional profiling

Broad-scale gene discovery by differential cDNA analysis, expressed sequence tag (EST) sequencing and cDNA-based microarrays allows global insights into cellular adaptation at the level of gene expression. In dermatophytes, such techniques were recently established and revealed the transcriptional response of these fungi under different biologically interesting and also pathogenicity-related conditions. A comprehensive *T. rubrum* Expression Database was launched by Wang *et al.* (2004, 2006), offering a platform for ESTs and cDNA microarray-based transcriptional profiles (http://www.mgc.ac.cn/TrED/). Documented in a number of publications, this approach resulted in the identification of *T. rubrum* genes, whose expression is linked to distinct developmental growth phases or the presence of selected drugs (Liu *et al.*, 2007; Yang *et al.*, 2007; Yu *et al.*, 2007; Zhang *et al.*, 2007, 2009). Broad transcriptional analyses were also performed in our work on *T. rubrum* and *A. benhamiae*, with a focus on genes putatively implicated in extracellular proteolysis. Herein, ESTs from *T. rubrum* grown on protein as the sole carbon and nitrogen source were analysed and used for the construction of a cDNA microarray containing at least 23 protease genes (Zaugg *et al.*, 2009). Major dermatophyte-secreted

keratinases have been known before and were correlated with the degradation of hard compact keratin (for a review, see Monod, 2008). Notably, dermatophytes were shown to secrete multiple serine proteases of the subtilisin family (Sub) as well as metalloproteases of the fungalysin family (Mep) [S8 and M36 family, respectively, in the MEROPS proteolytic enzyme database (http://merops.sanger.ac.uk)]. Microarray analysis during the growth of *T. rubrum* or *A. benhamiae* on soy and keratin protein confirmed the activation of particular *SUB* and *MEP* genes as well as genes encoding secreted exoproteases such as leucine aminopeptidases and dipeptidylpeptidases. In addition, other specifically induced factors playing a potential role in protein utilization were identified, including heat shock proteins, various transporters, metabolic enzymes, transcription factors and hypothetical proteins with unknown functions (Zaugg *et al.*, 2009; Staib *et al.*, 2010). Similar approaches were also supported by the analysis of suppression subtractive hybridization libraries, applied for the identification of novel dermatophyte genes specifically expressed by *T. rubrum* cells upon contact with keratin, in response to varying pH or to other environmental stimuli (Kaufman *et al.*, 2005; Baeza *et al.*, 2007; Maranhao *et al.*, 2007, 2009; Peres *et al.*, 2010; Silveira *et al.*, 2010). A comparative transcriptional analysis in the two closely related species *T. tonsurans* and *Trichophyton equinum* detected differential, species-specific expression levels of selected genes encoding secreted proteases upon growth on keratin (Preuett *et al.*, 2010).

## Gene expression profiling in *A. benhamiae* during infection

In order to unravel pathogenicity-related adaptation mechanisms of dermatophytes during infection, we explored the transcriptional response of the fungal cells in an animal model. For this approach, the zoophilic dermatophyte *A. benhamiae* was selected as an appropriate species for several reasons (Fig. 2). *Arthroderma benhamiae* is zoophilic and causes inflammatory cutaneous infections not only in humans but also in guinea-pigs, allowing the establishment of an animal model (Staib *et al.*, 2010). Under laboratory conditions, *A. benhamiae* grows relatively fast and produces abundant microconidia, single-nucleated round-oval cells that are useful for transformation. Cleistothecia formation further facilitates genetic analyses and allows to shed light on the basis of sexual development in dermatophytes. As a major additional prerequisite, the genome of our *A. benhamiae* strain, which had been isolated from a patient with highly inflammatory tinea faciei (Fumeaux *et al.*, 2004), has recently been decoded and annotated (Burmester *et al.*, 2011) (Fig. 2). Transcriptional analysis in *A. benhamiae* cells isolated during experimental cutaneous infection of guinea-pigs uncovered a distinct protease gene expression profile, which is essentially different from the pattern displayed during *in vitro* growth on keratin. Most notably, a differential expression of genes coding for members of the Sub and Mep protease families was detected. Instead of the

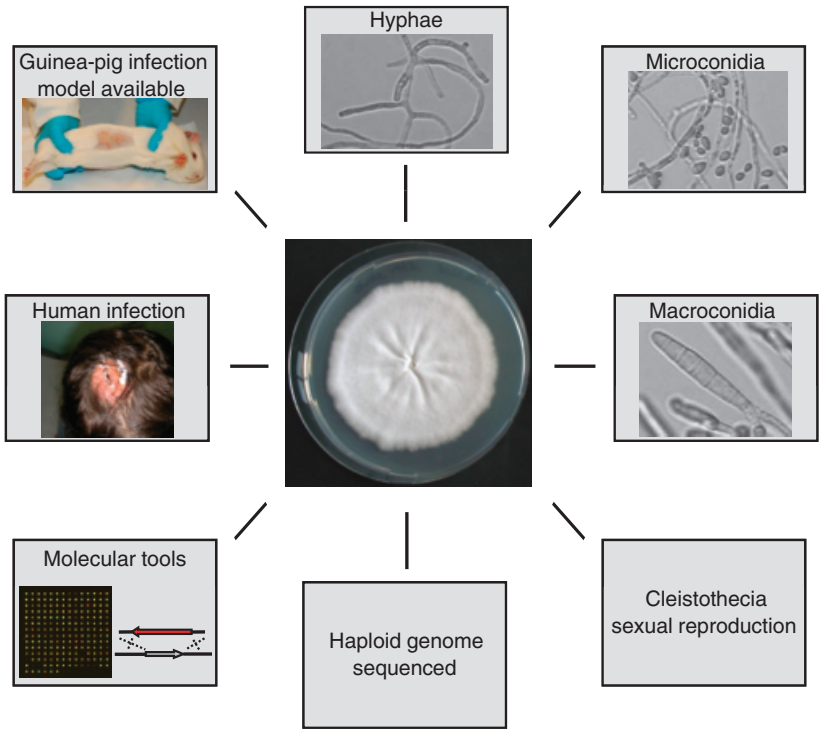


**Fig. 2.** Summary of the basic characteristics that make *Arthroderma benhamiae* a useful model for molecular research in dermatophytes. The centre shows a typical colony of *A. benhamiae* on Sabouraud glucose agar after 5 days of growth at 30 °C. Detailed explanations are given in the text.

major keratinase genes expressed *in vitro*, others were activated specifically during infection, suggesting functions that are not necessarily associated with the degradation of keratin. Future studies will address the strong *in vivo* activation of the gene encoding the serine protease Sub6, a known major allergen in the related dermatophyte *T. rubrum*. The broad *A. benhamiae in vivo* gene expression profile further revealed other putatively pathogenicity-related factors, whose role has to be studied by straightforward functional analysis. Other interesting, putatively pathogenicity-related dermatophyte genes have been identified recently in a broad transcriptome approach in *A. benhamiae* during the interaction with human keratinocytes (Burmester *et al.*, 2011).

## Transformation and gene targeting in dermatophytes

### Transformation and selection markers

In comparison with many other fungi, dermatophytes have been shown to be less amenable to genetic manipulation. As a result, site-directed mutagenesis in dermatophyte species has been evidenced only in a very small number of cases. This drawback is assumed to be a result of both low transformation frequency and inefficient homologous integration, processes that are indispensable for targeted genetic manipulations. The first successful transformation of a dermatophyte has been described in 1989 by Gonzalez *et al.* (1989) in *T. mentagrophytes* (Table 1). The transformation protocol applied was based on a standard protoplast/polyethylene glycol (PEG)-mediated procedure that has been established widely in filamentous fungi, for example *Aspergillus nidulans*, *Neurospora crassa* and others (for a review, see Fincham, 1989; Weld *et al.*, 2006). As a marker for the selection of *T. mentagrophytes* transformants, the system used the bacterial hygromycin B phosphotransferase gene *hph*. Plasmid DNA was stably integrated into the fungal genome with varying integration sites and numbers of insertions in the resulting transformants. Thereafter, no further attempts on dermatophyte transformation have been reported until 2004, when Kaufman *et al.* (2004) described PEG-mediated protoplast transformation and restriction-enzyme-mediated integration in *T. mentagrophytes*, using the *hph* gene as a selectable marker and the gene encoding the enhanced green fluorescent protein (eGFP) as a reporter. PEG-mediated transformation and transformant selection via hygromycin resistance was further demonstrated in *M. canis* (Yamada *et al.*, 2005, 2006; Vermout *et al.*, 2007) and *T. rubrum* (Fachin *et al.*, 2006; Ferreira-Nozawa *et al.*, 2006). Different other drugs/dominant markers have meanwhile been proven successful for the selection of transformants in *T. mentagrophytes*, i.e. two other aminoglycoside antibiotics/resistance genes, nourseothricin/*Streptomyces noursei* nourseothricin acetyltransferase gene *nat1* (Alshahni *et al.*, 2010) and geneticin (G-418)/*Escherichia coli* neomycin phosphotransferase gene *neo* (Yamada *et al.*, 2008). The latter marker as well as *hph* were also used successfully in *A. benhamiae* (Grumbt *et al.*, 2011). Besides PEG-mediated protoplast transformation, other techniques facilitating gene transfer were also meanwhile adopted in dermatophytes. A promising *Agrobacterium tumefaciens*-mediated transformation (ATMT) system was established recently for *T. mentagrophytes* (Yamada *et al.*, 2009b). ATMT has already strongly advanced functional genomics in various filamentous fungi before (for a

**Table 1.** Chronological overview of successful genetic transformation experiments in dermatophytes

| Species | Method | Resistance gene | Specific integration | References |
|---|---|---|---|---|
| *T. mentagrophytes* | PEG | *hph* | No | Gonzalez *et al.* (1989) |
| *T. mentagrophytes* | PEG/REMI | *hph* | No | Kaufman *et al.* (2004) |
| *T. mentagrophytes*/ *M. canis* | PEG | *hph* | No | Yamada *et al.* (2005) |
| *M. canis* | PEG | *hph* | Yes | Yamada *et al.* (2006) |
| *T. rubrum* | PEG | *hph* | Yes | Fachin *et al.* (2006) |
| *T. rubrum* | PEG | *hph* | Yes | Ferreira-Nozawa *et al.* (2006) |
| *M. canis* | PEG | *hph* | No | Vermout *et al.* (2007) |
| *T. mentagrophytes* | PEG | *neo* | No | Yamada *et al.* (2008) |
| *T. rubrum* | Electroporation | *hph* | No | Dobrowolska & Staczek (2009) |
| *T. mentagrophytes* | ATMT | *hph* | Yes/no | Yamada *et al.* (2009b) |
| *T. mentagrophytes* | ATMT | *hph/neo* | Yes | Yamada *et al.* (2009a) |
| *T. mentagrophytes* | ATMT | *nat1* | Yes | Alshahni *et al.* (2010) |
| *T. mentagrophytes* | ATMT | *hph/neo* | Yes | Alshahni *et al.* (2011) |
| *A. benhamiae* | PEG | *hph/neo* | Yes | Grumbt *et al.* (2011) |

REMI, restriction-enzyme-mediated integration; *hph*, hygromycin phosphotransferase gene; *neo*, neomycin phosphotransferase gene; *nat1*, nourseothricin acetyltransferase gene.

review, see Michielse *et al.*, 2005). Notably, Yamada and colleagues used the system for both random integration of T- (transferred) DNA and targeted insertion, for example disruption of the *areA*/*nit-2* gene. As another alternative transformation technique, electroporation of germinated conidia was applied in *T. rubrum*, allowing the random integration of *hph* and *eGFP* (Dobrowolska & Staczek, 2009). Although not many comparative data on transformation efficiency are available – some species have not even been addressed at all – different dermatophyte species appear to be more or less amenable to DNA uptake and/or stable integration. Therefore, transformation protocols established for a selected species are not necessarily transferable to another, but require precise modifications. From our own work, we know for example that our standard PEG-protocol for the efficient transformation of *A. benhamiae* was not directly applicable for *T. rubrum* or *M. canis*. The reasons for this observation are likely multifactorial, including differential protoplast stability, cell wall composition, microconidia production, etc.

## Targeted gene inactivation

Filamentous fungi are known to only poorly support site-directed insertion of linear DNA cassettes in the genome by homologous recombination, in contrast to yeasts such as *Saccharomyces cerevisiae* or the opportunistic pathogen *C. albicans*. Therefore, in filamentous fungi, identification of transformants with a desired genetic alteration has proven laborious in many cases. In order to circumvent this obstacle, parental strains were generated in diverse species that lack the nonhomologous end joining (NHEJ) recombination pathway, for example in *N. crassa* (Ninomiya *et al.*, 2004), *Aspergillus* spp. (da Silva Ferreira *et al.*, 2006; Krappmann *et al.*, 2006; Nayak *et al.*, 2006), and since recently, also in *T. mentagrophytes* (Yamada *et al.*, 2009a) and *A. benhamiae* (Grumbt *et al.*, 2011) (Table 1). Mutants deficient in NHEJ processes allow a strongly increased frequency of targeted insertions; however, an altered risk of unforeseen genetic variations cannot be excluded. In dermatophyte species, only a small number of genes have so far been analysed by targeted inactivation, for example *pacC* and *MDR2* in *T. rubrum* (Fachin *et al.*, 2006; Ferreira-Nozawa *et al.*, 2006), *Ku80*, *areA* and *Trim4* in *T. mentagrophytes* (Yamada *et al.*, 2009a, b), *areA* in *M. canis* (Yamada *et al.*, 2006) and *Ku70* and *AcuE* in *A. benhamiae* (Grumbt *et al.*, 2011). Interestingly, *A. benhamiae* has been shown in our work to allow efficient targeted gene deletion not only in a *ku70* mutant background but also in the wild-type strain. This has been demonstrated by the construction of mutants in malate synthase *AcuE*, *KU70* and other candidates (Grumbt *et al.*, 2011; M. Grumbt and P. Staib, unpublished data). The use of two different dominant selection markers, *hph* and *neo*, even allowed for the first time the site-directed complementation of knockout mutant strains. Because the deletion of *KU70* had no adverse effect on the virulence of *A. benhamiae* in a guinea-pig infection model, both the wild type and the *ku70* mutant appear to be suitable parental strains for future pathogenicity research. In general, isogenic strain construction is assumably facilitated in species such as *A. benhamiae* and *T. mentagrophytes* by the fact that they easily allow the production of abundant single nucleated cells in the form of microconidia as a starting material.

## RNA silencing

RNA interference, originally described in the nematode *Caenorhabditis elegans*, is based on a cellular process by which an introduced double-stranded RNA induces the degradation of specific mRNAs of interest (Fire *et al.*, 1998). RNA silencing was widely applied as an efficient tool to address gene function in multiple research areas, especially when conventional site-directed gene inactivation is difficult or, due to knockout lethality, impossible. As another advantage, the technique offers the possibility to inhibit several genes at the same time, a characteristic that might be useful for the functional analysis of homologous genes within large families, for example those encoding secreted endoproteases in dermatophytes. Here, the system was first established by Vermout *et al.* (2007) by the construction of *M. canis* transformants in which the expression of genes encoding secreted proteases Sub3 and dipeptidyl peptidase IV, respectively, was suppressed. Using the *SUB3* RNA-silenced strain, the authors revealed a contribution of this protease in the adherence of *M. canis* to feline epidermis, whereas a function in epidermal invasion and virulence of the fungus during cutaneous guinea-pig infection was not assigned (Baldo *et al.*, 2010).

## Genome sequencing projects

Given the fact that powerful tools have meanwhile become available for the genetic manipulation of dermatophytes, the advent of dermatophyte genome sequencing projects now offers a fundamental basis for future research. Annotated genome sequences of seven different dermatophyte species have become available recently (http://www.broadinstitute.org/annotation/genome/dermatophyte_comparative/MultiHome.html), provided by projects headed by the Broad Institute (Cambridge) and the Hans Knoell Institute (Jena, Germany), respectively. The latter institution has recently published the first report on dermatophyte genomes, presenting a comparative study on the two closely related zoophilic, human pathogenic species *A. benhamiae* (major reservoir are guinea-pigs) and *T. verrucosum* (major

reservoir are cattle) (Burmester *et al.*, 2011). The genome sequences identified were compared not only with each other but also with those of other species of the *Onygenales*, i.e., *Coccidioides posadasii* and *Coccidioides immitis*, and with the mould *A. fumigatus*. The 22–23 Mb genomes of *A. benhamiae* and *T. verrucosum*, containing 7980 and 8024 predicted protein-encoding genes, respectively, were found to be smaller than those of *Aspergillus* (e.g. 28 and 37.3 Mb for *Aspergillus clavatus* and *Aspergillus niger*, respectively), *Coccidioides* spp. (27–29 Mb) or *Histoplasma capsulatum* (30–39 Mb). Special attention was paid not only to the analysis of genes that are putatively associated with host adaptation, for example genes encoding secreted proteases. Genes involved in the biosynthesis of secondary metabolites and mating were also found to be of future interest (Burmester *et al.*, 2011). Additional insights are expected from the envisaged genome comparison including the other five sequenced human pathogenic dermatophyte species. The species selection was based on different biological parameters and pathogenicity-related hypotheses (White *et al.*, 2008), and the basic traits of the selected strains such as growth rate and resistance to diverse antibiotics were already monitored (Achterman *et al.*, 2011). Because these species encompass anthropophilic (*T. rubrum*, the most common inducer of dermatophytosis in humans worldwide; *T. tonsurans*, often associated with tinea capitis in America), zoophilic (*T. equinum*, associated with horses; *M. canis*, associated with cats and dogs) and geophilic (*M. gypseum*) dermatophytes, a comparative genome analysis will, among other topics, address factors that are potentially involved in host preference, adaptation during chronic vs. inflammatory infection and saprophytic growth.

## Conclusion

An increasing, lively interest in the molecular biology of dermatophytes combined with the establishment of fundamental genetic approaches has strongly advanced the research in these filamentous fungi. Basic prerequisites have been launched, such as genome sequencing projects, expression profile data sets and efficient targeted gene inactivation techniques. Nevertheless, molecular research is still preliminary in these genetically less amenable microorganisms. Therefore, further efforts have to be undertaken for the improvement of existing and the establishment of additional genetic tools and methodologies. Such efforts will be worthwhile, given the fact that dermatophytoses are widespread and of particular clinical interest. Using the available techniques, now fundamental questions can be addressed in dermatophytes, related to the pathogenicity as well as general host and environmental adaptation mechanisms, sexual development, basic biology and evolution.

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