Phylogenetic analysis of *Trichophyton mentagrophytes* human and animal isolates based on MnSOD and ITS sequence comparison

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Dermatophytes are keratinophilic fungi able to infect keratinized tissues of human or animal origin. Among them, *Trichophyton mentagrophytes* is known to be a species complex composed of several species or variants, which occur in both human and animals. Since the *T. mentagrophytes* complex includes both anthropophilic and zoophilic pathogens, accurate molecular identification is a critical issue for comprehensive understanding of the clinical and epidemiological implications of the genetic heterogeneity of this complex. Here, 41 *T. mentagrophytes* isolates from either human patients (14 isolates) or animals (27 isolates) with dermatophytosis were prospectively isolated by culture and identified on morphological bases at the University Hospital Centres of Lille and Poitiers, and the Veterinary School of Alfort, respectively. The isolates were differentiated by DNA sequencing of the variable internal transcribed spacer (ITS) regions flanking the 5.8S rDNA, and of the housekeeping gene encoding the manganese-containing superoxide dismutase (MnSOD), an enzyme which is involved in defence against oxidative stress and has previously provided interesting insight into both fungal taxonomy and phylogeny. ITS1-ITS2 regions and MnSOD sequences successfully differentiate between members of the *T. mentagrophytes* complex and the related species *Trichophyton rubrum*. Whatever the phylogenetic marker used, members of this complex were classified into two major clades exhibiting a similar topology, with a higher variability when the ITS marker was used. Relationships between ITS/MnSOD sequences and host origin, clinical pattern and phenotypic characteristics (macroscopic and microscopic morphologies) were analysed.

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INTRODUCTION

Dermatophytes are keratinophilic fungi able to infect keratinized tissues of human or animal origin, leading to infections that are mainly restricted to the corneocytes of the skin, hair and nails. These filamentous fungi are usually

identified on the basis of clinical features and isolation patterns together with conidial morphology, and sometimes with physiological characters, such as the hair perforation and urease tests. In some cases, morphological identification can be difficult or uncertain because there is considerable variation and pleomorphism among isolates of the same species. In the last decade, genotyping approaches have proven to be useful for solving problems of dermatophyte taxonomy, as well as enhancing the reliability and speed of dermatophytosis diagnosis (De Hoog *et al.*, 1998; Faggi *et al.*, 2001, 2002; Graser *et al.*,

Abbreviations: ITS, internal transcribed spacer; ML, maximum likelihood; MnSOD, manganese-containing superoxide dismutase; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the MnSOD and ITS sequences determined in this work are given in the text and Fig. 1.

2006; Jousson *et al.*, 2004; Kano *et al.*, 2003; Kardjeva *et al.*, 2006; Liu *et al.*, 2000; Mochizuki *et al.*, 1990, 1999; Ninet *et al.*, 2003; Weitzman & Summerbell, 1995).

Members of the *Trichophyton mentagrophytes* species complex are common agents of human dermatophytosis in Europe (Kardjeva *et al.*, 2006; Monod *et al.*, 2002), invading skin layers (Kaufman *et al.*, 2004) and causing superficial infections, such as tinea pedis or athlete's foot (De Hoog *et al.*, 1998; Weitzman & Summerbell, 1995), with an increased incidence since 1965 (Kardjeva *et al.*, 2006). The species included in this anthro-po-zoophilic complex, which were first defined on the basis of morphological features and mating-type studies, have more recently been investigated using molecular methods, revealing an organization more complex than expected. These molecular approaches have been based primarily on the G+C content of chromosomal DNA (Davison *et al.*, 1980), total DNA homology (Davison & Mackenzie, 1984), restriction fragment length polymorphism analysis of mitochondrial DNA (mtDNA) (Mochizuki *et al.*, 1990, 1996), arbitrarily primed PCR (AP-PCR) (Liu *et al.*, 2000), random amplification of polymorphic DNA (RAPD) analysis (Kac *et al.*, 1999; Liu *et al.*, 1997; Mochizuki *et al.*, 1997) and PCR fingerprinting (Faggi *et al.*, 2001; Graser *et al.*, 1999b). More recently, sequence analysis of the rDNA regions (Graser *et al.*, 1999a; Harmsen *et al.*, 1995; Makimura *et al.*, 1998, 1999; Mochizuki *et al.*, 1999, 2003) and, in particular, analysis of internal transcribed spacer (ITS) regions (Graser *et al.*, 1999a; Makimura *et al.*, 1998, 1999; Mochizuki *et al.*, 1999), which appeared more suitable than the gene coding for the small-subunit rRNA (18S rRNA) (Harmsen *et al.*, 1995), were used successfully to evaluate the phylogenetic relationships within the *T. mentagrophytes* complex. These accurate molecular studies emerged as a critical issue for comprehensive understanding of the clinical and epidemiological implications of the genetic heterogeneity of *T. mentagrophytes* and resulted in the re-establishment of three if not four species: *Trichophyton interdigitale* close to the teleomorph *Arthroderma vanbreuseghemii*, *T. mentagrophytes* close to the teleomorph *A. simii*, and *T. erinacei*, plus another *Trichophyton* anamorph, both close to the teleomorph *A. benhamiae*, which includes both European-American and African races (Graser *et al.* 1999b; Probst *et al.*, 2002; Takahashi *et al.*, 2003; Nenoff *et al.*, 2007). Among these four species, two were rooted in typification, according to the criteria of the International Code of Botanical Nomenclature: *T. mentagrophytes* (strain CBS 318.56) and *T. interdigitale* (strain CBS 428.63) (Graser *et al.*, 1999b).

Molecular analysis using protein-encoding gene sequence approaches are poorly developed among the genera of dermatophyte species. The chitin synthase 1 gene and fungalsin gene family are the unique protein-encoding genes that have been sequenced to provide information for the identification of *Trichophyton* species and the understanding of their evolution (Jousson *et al.*, 2004; Kano *et al.*,

1998, 2003). In the present study, we attempted to evaluate the relationships between members of the *T. mentagrophytes* complex isolated from both human beings and animals using a housekeeping gene encoding the manganese-containing superoxide dismutase (MnSOD), which is involved in cell defence against endogenous and exogenous reactive oxygen species, and which has previously provided interesting insights into both fungal taxonomy, contributing to the differentiation of *Pneumocystis* species (Denis *et al.*, 2000), and phylogeny, leading to a complex evolutionary scenario of the MnSOD gene with several gene duplication events (Fréalle *et al.*, 2005, 2006).

As the *T. mentagrophytes* complex includes both anthropophilic and zoophilic pathogens, we included both human and animal isolates (natural and occasional hosts), with the aim of analysing their phylogenetic distribution within the four species of the *T. mentagrophytes* complex. Using this phylogenetic distribution, we explored the potential association of each species with specific hosts, and with characteristic and stable morphological features, trying to get reliable criteria for the routine identification of these species. The polymorphism of the ITS1 and ITS2 non-coding regions flanking the DNA sequence encoding the 5.8S rRNA was also analysed and compared with the MnSOD sequence results.

METHODS

Fungal strains. Forty-one *T. mentagrophytes* isolates from either human patients with dermatophytosis (14 isolates) or animals with or without visible lesions (27 isolates) were prospectively isolated by culture and identified on the basis of their morphological features at the University Hospital Centres of Lille and Poitiers, and the Veterinary School of Alfort, respectively, from May 2002 to March 2004. These isolates were compared to the *T. mentagrophytes* neotype (CBS 318.56), and to the following three strains: *T. interdigitale* (TMC-42, Lille Pasteur Institute), a zoophilic strain of *T. interdigitale* (TMC-43, Lille Pasteur Institute) and *T. erinacei* (TMC-44, Lille Pasteur Institute), for which the species identification was confirmed at the Pasteur Institute of Paris Mycology Department. These three strains (TMC-42–44) were used as standard strains in this study. *T. interdigitale* TMC-42, *T. interdigitale* TMC-43, and one representative of every novel genotype were deposited in the CBS collection. Collection numbers are indicated in Table 1. All the *T. mentagrophytes* isolates and strains were analysed at the molecular level using *T. rubrum* TR-104 (Lille Pasteur Institute) as an outgroup.

All strains were grown at 30 °C on Sabouraud's agar medium with kanamycin and with kanamycin plus cycloheximide for 3 weeks. They were identified on the basis of microscopy and colony characteristics and were classified using the following criteria: (i) clinical criteria (body localization, associated or not with clinical signs or symptoms of dermatophytosis), (ii) cultural aspects (texture, front and reverse colour of the colonies), and (iii) microscopic features (subspherical or clavate microconidia, macroconidia, spiral hyphae, and dense branchlets at right angles) (Graser *et al.*, 1999b; Mochizuki *et al.*, 1996, 2003; Ninet *et al.*, 2003). Morphological data for the isolates, and their origins, are listed in Table 1.

DNA extraction. DNA was extracted from a small amount of mycelium grown on Sabouraud dextrose agar by a rapid preparation method (Fréalle *et al.*, 2005, 2006). Each isolate was treated separately,

Table 1. Clinical and culture data of the isolates of *Trichophyton* spp. used in the study

TMC or CBS no.*	General and clinical data		Macroscopic and microscopic features of Sabouraud agar culture				
	Origin†	Source (sex, age, symptomatology)‡	Colony morphology§	Microscopic features			
				Conidia criteria		Mycelium criteria	
				Microconidia	Macroconidia	Spiral hyphae	Branchlets at right angles
Clade I							
<i>Subclade 1, Group A</i>							
TMC-3 (H) =CBS 121072	Lille	F, 15 y, Tinea pedis	Downy with yellow-orange to brown reverse	Subspherical	—	—	—/+
TMC-6 (H)	Lille	M, 41 y, Onychomycosis	Powdery with yellow to orange reverse	Subspherical to ovoid	—	—	++
TMC-10 (H)	Lille	F, 37 y, Skin	Powdery with yellow to orange reverse	Subspherical to ovoid	—	+++	—/+
TMC-11 (H)	Poitiers	F, 14 y, Tinea pedis	Downy with yellow-orange to brown reverse	Subspherical	—	++	—
TMC-12 (H)	Poitiers	M, 68 y, Tinea pedis	Downy to powdery with yellow-orange reverse	Subspherical	+	++	—/+
TMC-13 (H)	Poitiers	M, 39 y, Onychomycosis	Downy to powdery with yellow-orange reverse	Subspherical	+	++	—
TMC-14 (H)	Poitiers	M, 78 y, Tinea pedis	Downy to powdery with yellow-orange reverse	ND	ND	ND	ND
TMC-18 (A)	ENVA	M, Guinea pig	Powdery with brown to reddish-purple reverse	Subspherical	++	+++	—/+
TMC-25 (A)	ENVA	M, 3 m, Guinea pig	Powdery with orange to brown reverse	Subspherical	—	—	—/+
TMC-7 (H) =CBS 121069	Lille	M, 56 y, Tinea corporis/inflammatory lesion	Powdery with brown to reddish-purple reverse	Subspherical	++	+	—
TMC-40 (A)	ENVA	F, 6 y, Dog	Downy with yellow to orange reverse	Subspherical	—	+++	—
TMC-41 (A)	ENVA	F, 2 y, Pig	Powdery with brown to reddish-purple reverse	Subspherical to ovoid	+++	+++	+
<i>Subclade 1, Group B</i>							
TMC-32 (A)	ENVA	Chinchilla	Downy with yellow to orange reverse	Subspherical	+	—	++
TMC-33 (A) =CBS 121065	ENVA	Chinchilla	Powdery with orange to brown reverse	Subspherical	—	—	++
TMC-35 (A)	ENVA	Chinchilla	Powdery to downy with brown to reddish-purple reverse	Subspherical	—	—	—
TMC-36 (A) =CBS 121064	ENVA	Mouse	Powdery with orange to brown reverse	Subspherical	—	++	—
TMC-37 (A)	ENVA	F, 1 y, Mouse	Powdery with orange to brown reverse	Subspherical	—	+++	—
TMC-38 (A)	ENVA	Mouse	Powdery with orange to brown reverse	Subspherical	—	—	—
TMC-4 (H) =CBS 121070	Lille	F, 18 y, Tinea corporis	Powdery with yellow-orange to brown reverse	Subspherical to ovoid	—	+++	—/+
TMC-8 (H)	Lille	M, 12 y, Skin/inflammatory lesion	Powdery with brown reverse	Subspherical	++	++	—

Table 1. cont.

TMC or CBS no.*	General and clinical data		Macroscopic and microscopic features of Sabouraud agar culture				
	Origin†	Source (sex, age, symptomatology)‡	Colony morphology§	Microscopic features			
				Conidia criteria		Mycelium criteria	
				Microconidia	Macroconidia	Spiral hyphae	Branchlets at right angles
Clade II							
TMC-1 (H)	Lille	F, 7 y, Skin of ear	Downy with yellow-orange to brown reverse	Clavate	—	—	+ +
TMC-2 (H)	Lille	M, 11 y, Skin/ inflammatory lesion	Powdery with yellow to orange reverse	Clavate	—	—	+ +
TMC-5 (H)	Lille	F, 6 y, Tinea corporis/ inflammatory lesion	Powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	—/ +	+ + +
TMC-9 (H)	Lille	F, 10 y, Tinea capitis/ inflammatory lesion	Downy to powdery with yellow to orange reverse	Subspherical to ovoid	—	—	+ +
TMC-15 (A) =CBS 121071	ENVA	M, Guinea pig	Downy to powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	—	+ + +
TMC-16 (A)	ENVA	M, Guinea pig	Downy to powdery with brown to reddish-purple reverse	Subspherical	—	—	+ +
TMC-17 (A)	ENVA	F, Guinea pig	Downy to powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	—	+ + +
TMC-19 (A)	ENVA	F, Guinea pig	Powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	+	+
TMC-20 (A)	ENVA	F, 2 m, Guinea pig	Downy to powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	—	+ + +
TMC-21 (A)	ENVA	F, 3 m, Guinea pig	Powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	+	—
TMC-22 (A)	ENVA	F, 3 y, Guinea pig	Powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	—	+ + +
TMC-23 (A)	ENVA	Guinea pig	Downy to powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	—	+ + +
TMC-24 (A)	ENVA	M, Guinea pig	Downy to powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	—	+ + +
TMC-26 (A)	ENVA	M, 3 m, Rabbit	Downy to powdery with orange reverse	Subspherical to ovoid	—	—	+ + +
TMC-27 (A)	ENVA	M, 3 m, Rabbit	Downy to powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	+	+ +
TMC-28 (A)	ENVA	F, 3 m, Rabbit	Downy to powdery with yellow to orange reverse	ND	ND	ND	ND
TMC-29 (A)	ENVA	M, 4 m, Rabbit	Downy with brown to reddish-purple reverse	Subspherical	—	—	+ +
TMC-30 (A)	ENVA	Rabbit	Powdery with orange to brown reverse	Clavate	+ + +	—	+ + +
TMC-31 (A)	ENVA	F, 4 m, Rabbit	Downy with brown to reddish-purple reverse	Subspherical to ovoid	+	—	+ + +
TMC-34 (A) =CBS 121067	ENVA	Chinchilla	Downy with yellow to orange reverse	Subspherical	—	—	—

Table 1. cont.

TMC or CBS no.*	General and clinical data		Macroscopic and microscopic features of Sabouraud agar culture				
	Origin†	Source (sex, age, symptomatology)‡	Colony morphology§	Microscopic features			
				Conidia criteria		Mycelium criteria	
				Microconidia	Macroconidia	Spiral hyphae	Branchlets at right angles
TMC-39 (A)	ENVA	M, 2 y, Dog	Downy to powdery with brown to reddish-purple reverse	Subspherical to ovoid	—	+	+++
Standard strains identified at Pasteur Institute of Paris							
TMC-42: <i>T. interdigitale</i> = CBS 121066		Human	Downy with yellow-orange to brown reverse	Subspherical	—	++	—/+
TMC-43: zoophilic strain of <i>T. interdigitale</i> = CBS 121068		Unspecified	Powdery with brown to reddish-purple reverse	Subspherical	+/+++	++	—
TMC-44: <i>T. erinacei</i>		Unspecified	Downy to powdery with yellow to orange reverse	Clavate	+++	—	+++

*A TMC number was attributed to each *T. mentagrophytes* isolate included in our study. *T. interdigitale* (TMC-42), *T. interdigitale* zoophilic strain (TMC-43), and one representative of every genotype were deposited in the CBS Collection. CBS numbers of these isolates are indicated in bold. (H), human isolates; (A), animal isolates.

†ENVA, Ecole Nationale Vétérinaire d'Alfort.

‡M, male; F, female. When host age is known: y, years; m, months.

§Morphology on Sabouraud agar medium incubated at 30 °C for 3 weeks.

||Absence or presence of macroconidia, spiral hyphae, and mycelium branchlets at right angles were noted as follows: absence (—), few (+), moderate (++) or abundant (+++). ND, Not determined.

under a laminar-airflow hood to avoid contamination. Briefly, fresh mycelium (approx. 1 cm²) was collected and put into an Eppendorf tube containing 1 ml distilled water. After centrifugation at 8000 r.p.m. for 2 min, DNA was extracted according to the protocol of the QIAamp DNA minikit column (Qiagen), and eluted with 200 µl distilled water.

Sequencing of the MnSOD and ITS loci. A 420 or 421 bp fragment of the MnSOD gene was amplified by PCR using the following pair of specific primers: Tm-SODf (5'-CATCATCAAACCTACGTCAAC-3') and Tm-SODr (5'-TAGTAGGCGTGCTCCCAGG-3'), designed using the *T. mentagrophytes* partial MnSOD sequence (GenBank accession no. AY625498) determined in a previous study (Fréalle *et al.*, 2005, 2006). Amplification reactions were performed in a final volume of 50 µl containing 6 µl template DNA, 5 µl reaction buffer (10×), 1.5 µM MgCl₂, 200 µM of each dNTP, 0.8 µM of each primer and 2 units *Taq* polymerase (AmpliTaq Gold, Roche Molecular Biochemicals). The PCR cycling protocol was performed in a PTC-200 DNA Engine (MJ Research) as follows: one initial denaturation step of 3 min at 94 °C, followed by 40 cycles consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 47 °C and extension for 1 min at 72 °C, followed by a final extension for 10 min at 72 °C.

The ribosomal ITS regions were amplified according to published methods (Graser *et al.*, 1999a, b). Briefly, amplification reactions were performed in a final volume of 100 µl containing 6 µl template DNA, 10 µl reaction buffer, 1.5 µM MgCl₂, 200 µM of each dNTP, 0.8 µM of each universal primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and 3 units *Taq*

polymerase (AmpliTaq Gold, Roche Molecular Biochemicals). Each PCR mixture was heated to 94 °C for 3 min, and the PCR was then performed under the following conditions: 20 cycles consisting of 94 °C for 35 s, 54 °C for 55 s, 72 °C for 45 s, increasing the extension time by 4 s on each cycle, and 10 cycles consisting of 94 °C for 45 s, 54 °C for 55 s, 72 °C for 2 min, increasing the extension time by 4 s on each cycle, with a final extension at 72 °C for 6 min.

Two per cent agarose gel electrophoresis was performed to examine the quality of the PCR products. Forty microlitres of each PCR product was purified with the QIAEX II Gel Extraction kit (Qiagen). Final DNA concentration was evaluated by measuring A₂₆₀. Both strands of purified PCR products (5–10 µl; 700 ng) were directly sequenced by using Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) with primers Tm-SODf/Tm-SODr or ITS1/ITS4 depending on the targeted gene (MnSOD gene or ITS region respectively). Sequences were run on an automated ABI Prism 377 DNA sequencer (Applied Biosystems), according to the manufacturer's instructions. For each PCR product, a consensus nucleotide sequence was determined using BioEdit software (Tippmann, 2004) and significant sequence identity with known fungal MnSOD gene or ITS region sequences was displayed by using the BLAST standard nucleotide–nucleotide local alignment search tool (NCBI; <http://www.ncbi.nlm.nih.gov/BLAST/>). *T. rubrum* isolate TR-104 showed complete sequence identity to *T. rubrum* CBS 392.58 at the ITS region locus (sequence accession number Z97993; Graser *et al.*, 1999a).

Determination of the deduced amino acid MnSOD sequences. Amino acid sequences were deduced from each MnSOD nucleotide

consensus sequence. The *T. mentagrophytes* MnSOD sequences, as well as the *T. rubrum* MnSOD sequence, had an open reading frame composed of two exons and one intron whose position had been previously identified by canonical 5' (GT) and 3' (AG) intron/exon boundaries (Parker & Patterson, 1987), and confirmed by RT-PCR (Fréalles *et al.*, 2006).

Phylogenetic analysis. The partial MnSOD gene sequences and the corresponding deduced amino acid sequences were aligned using CLUSTAL W as implemented in the BioEdit program (Tippmann, 2004). Using the same program, ITS sequences of the same isolates were aligned with 24 ITS sequences of the *T. mentagrophytes* complex and *T. rubrum* retrieved from GenBank. Accession numbers of the sequences included in the ITS dataset are listed in Fig. 1.

The phylogenetic trees were constructed by the neighbour-joining (NJ) and maximum-likelihood (ML) methods, using the PHYLIP package (Felsenstein, 1997). The reliability of internal branches was assessed using the bootstrap method implemented in the PHYLIP program, with 1000 (NJ) and 100 (ML) replicates. Phylogenetic trees were rooted with *T. rubrum* as an outgroup, and edited using TreeView (Page, 1996).

Nucleotide sequence accession numbers. The MnSOD and ITS region sequences determined in this study were submitted to the GenBank nucleotide sequence database. The ITS region sequence accession numbers (DQ786656 to DQ786699) are indicated in Fig. 1. The MnSOD sequence accession numbers are DQ786700 to DQ786744: DQ786700 to DQ786740 for isolates TMC-1 to TMC-41, DQ786741 for the *T. interdigitale* standard strain (TMC-42), DQ786742 for the zoophilic strain of *T. interdigitale* (TMC-43), DQ786743 for the *T. erinacei* standard strain (TMC-44), EF614237 for *T. mentagrophytes* strain CBS 318.56, and DQ786744 for the isolate of *T. rubrum* (TR-104).

RESULTS AND DISCUSSION

Epidemiology and ecology

Considering our human isolates ($n=14$), the sex distribution ratio (M/F) was 1.1, lower than found in a previous study (Contet-Audonneau *et al.*, 2001); the age population distribution, with a majority of 10–19 year-old patients, was in accordance with previous results (Contet-Audonneau *et al.*, 2001). We found a majority of glabrous skin manifestations (tinea corporis) and athlete's foot (tinea pedis), in accordance with previous reports in Europe (Nowicki *et al.*, 1994) (Table 1).

The hosts of our animal isolate sample ($n=27$) were mainly rodents ($n=18$): mice, guinea pigs and chinchillas. This host population is mainly composed of exotic pets that have been recently introduced into Europe, including France, influencing the epidemiology of the *T. mentagrophytes* complex in these regions (Summerbell *et al.*, 2002; Weitzman & Summerbell, 1995) (Table 1).

Analysis of MnSOD sequences

All the nucleotide sequences analysed in this study exhibited a high degree of identity (>94.5%) with genes encoding *T. mentagrophytes* tetrameric MnSOD sequences available in databases (Fréalles *et al.*, 2006). Fragment length

was 420 or 421 bp, with an open reading frame composed of two exons separated by a 58 or 59 bp intron.

Based on BLAST searches at the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>), the corresponding deduced SOD amino acid sequences exhibited a higher degree of identity to the tetrameric MnSODs than to the other types of SODs, suggesting that they all belong to the class of tetrameric MnSODs (80% identity with the MnSOD amino acid sequence of *Coccidioides immitis*: EAS36233). Although our fungal SOD protein sequences were partial, they displayed almost all features of tetrameric MnSODs, including the residue Phe²⁷ systematically encountered in tetramers and never in dimers, and the manganese-specific residues Gly³⁰, Gly³¹, Phe³⁸, Gln¹⁰⁷ and Asp¹⁰⁸ (numbering as in sequences submitted to GenBank; Parker & Blake, 1988). When alignment was performed using CLUSTAL W, we identified 29 mutations (including one insertion/deletion) at the MnSOD locus for isolates restricted to genotypes II, III (isolate TMC-34), IV (*T. erinacei* TMC-44) and V (*T. mentagrophytes* neotype CBS 318.56) (Table 2). Most nucleotide mutations were localized within the intron, or were exon silent substitutions. Only two non-synonymous mutations (amino acid residues at positions 70 and 95, Table 2) were found in isolates of MnSOD genotypes II, III, IV and V.

Phylogenetic analysis

Phylogenetic analysis was performed for both the MnSOD and ITS datasets (Fig. 1). All sites of the MnSOD nucleotide and protein sequences could be unambiguously aligned, yielding 421 nucleotide positions and 120 amino acid sites respectively. The ITS sequences included mainly ITS1 and ITS2 regions, and the 5.8S rDNA gene. Regions of ambiguous alignment were removed, yielding 637 nucleotide sites for the dataset analysis. Given the low variability observed among MnSOD sequences, the MnSOD phylogenetic study resulted in only five groups, corresponding to the five genotypes described in Table 2 and shown in the ITS tree (Fig. 1).

In accordance with previous results (Graser *et al.*, 1999a, b; Makimura *et al.*, 1998), members of the *T. mentagrophytes* complex fell into two major closely related clades, emerging at a considerable distance from *T. rubrum*, which was used as an outgroup. The *T. mentagrophytes* complex showed marked genetic heterogeneity, as previously described (Graser *et al.*, 1999a, b; Makimura *et al.*, 1998), and the topology resulting from ITS sequences showed higher variability than that based on MnSOD sequence polymorphism (up to 10.6% for ITS vs 5.5% for MnSOD nucleotide sequences).

The *T. mentagrophytes* complex isolates and strains fell into five groups (indicated as 'MnSOD genotype' in Fig. 1) when MnSOD DNA sequences were analysed. The MnSOD sequence differences between isolates and/or the standard strains were generally single-nucleotide polymorphisms. Similar findings were made previously when other target

* MnSOD genotype (I, II, III, IV or V) is indicated for each TMC isolate

** ITS genotypes I, II, III, IV named according to Ninet *et al.* (2003)

genes were used: Ninet *et al.* (2003) were able to distinguish three *T. mentagrophytes* genotypes on the basis of two 28S rDNA polymorphic sites, and Kano *et al.* (1998, 2003) were

able to identify dermatophyte species with high sequence identity (99%) between reference strains and clinical isolates using a chitin synthase 1 based PCR assay.

Fig. 1. Rooted NJ tree of the *T. mentagrophytes* complex inferred from ITS sequences. *T. rubrum* served as an outgroup. ITS sequence accession numbers are given after each strain number. Bootstrap NJ and ML values are given as percentages near the individual nodes; values of <50% are shown as *. The evolutionary distances between organisms are indicated by the horizontal branch lengths (scale bar: 0.01 substitutions per base pair), which reflect the number of nucleotide substitutions per site along the branches from node to end point. The percentages of bootstrap samplings supporting the interior branches are noted. MnSOD sequence accession numbers are: DQ786700 to DQ786740 for isolates TMC-1 to TMC-41, DQ786741 for the *T. interdigitale* standard strain (TMC-42), DQ786742 for the zoophilic strain of *T. interdigitale* (TMC-43), DQ786743 for the *T. erinacei* standard strain (TMC-44), EF614237 for *T. mentagrophytes* strain CBS 318.56, and DQ786744 for *T. rubrum* clinical isolate TR-104. For each TMC isolate, the corresponding MnSOD genotype (I, II, III, IV or V) is indicated on the right.

Similarly to the previously described 15% variation at the ITS region locus within the *T. mentagrophytes* complex (Graser *et al.*, 1999a), ITS sequences from our isolates and standard strains reached up to 10.6% divergence, and fell into two clades. Clade I was composed of two subclades (Fig. 1). Subclade 1 included *T. interdigitale* CBS 558.66 (close to the *T. interdigitale* neotype described by Graser *et al.*, 1999b), the zoophilic *T. interdigitale* strain TMC-43 and *A. vanbreuseghemii* CBS 646.73, and corresponded to the '*T. interdigitale*/*A. vanbreuseghemii*' subclade. Interestingly, isolates within this subclade showed high variability, yielding a subdivision into two main groups: group A, with species closer to *T. interdigitale* neotype, and group B, a mainly zoophilic cluster, which also contained *A. vanbreuseghemii* CBS 646.73. In subclade 2, the *T. mentagrophytes* neotype CBS 318.56 (group C) was closer to *A. simii* (group D), as previously described (Graser *et al.*, 1999b). Clustering of the *T. mentagrophytes* neotype and *A. simii* within clade I suggested that the *T. mentagrophytes* neotype is closer to *T. interdigitale* than to *T. erinacei*, in accordance with previous data (Graser *et al.*, 1999b). Clade II contained all *A. benhamiae*/*T. erinacei* database sequences (subclades 3, 4 and 5). As previously described (Makimura *et al.*, 1998; Summerbell *et al.*, 1999), ITS sequence analysis clearly distinguished the American-European from the African *A. benhamiae*, which was on a separate branch (subclade 5). The *T. erinacei*/American-European *A. benhamiae* subclade

(subclade 3) contained two groups: group E, which included only *T. erinacei* isolates [our *T. erinacei* standard strain TMC-44; *A. benhamiae* IFM 50998 and IFM 48154 are *T. erinacei* strains, isolated from hedgehog (Takahashi *et al.*, 2003)], and group F, the American-European *A. benhamiae*. Regarding the recent study of Takahashi *et al.* (2003), which confirmed morphological, physiological and genetic similarity among the hedgehog-borne isolates, forming a distinct mating group from American-European and from African races of *A. benhamiae* (none or partial fertility), group E corresponded to those hedgehog-borne isolates belonging to an independent variety which should be identified as '*A. benhamiae* var. *erinacei*' (Takahashi *et al.*, 2003). All our human and animal isolates ITS sequences belonging to clade II showed low variability and clustered in the '*Trichophyton* anamorph close to *A. benhamiae*' subclade (subclade 4, Fig. 1).

Correlation between TMC isolate distribution and host species/morphology/clinical pattern

TMC isolates were related either to *T. interdigitale*/*A. vanbreuseghemii* (clade I, subclade 1) or to '*Trichophyton* anamorph close to *A. benhamiae*' (clade II, subclade 4); none clustered with the *T. mentagrophytes* neotype (clade I, subclade 2), or with *T. erinacei* (clade II, subclade 3), except for our *T. erinacei* standard strain TMC-44. Within

Table 2. Position of the polymorphic sites on the alignment of *T. mentagrophytes* MnSOD partial nucleotide and protein sequences

The 'genotype I' sequence was found in *T. mentagrophytes* clinical isolates TMC-3, 4, 6–8, 10–14, 18, 25, 32, 33, 35–38, 40 and 41, the *T. interdigitale* standard strain (TMC-42) and the zoophilic strain of *T. interdigitale* (TMC-43). The 'genotype II' sequence was found in *T. mentagrophytes* clinical isolates TMC-1, 2, 5, 9, 15–17, 19–24, 26–31 and 39. The 'genotype III' sequence was found in *T. mentagrophytes* clinical isolate TMC-34. The 'genotype IV' sequence was found in the *T. erinacei* (TMC-44) standard strain. The 'genotype V' sequence was found in the *T. mentagrophytes* neotype (CBS 318.56). These five genotypes resulted in only two deduced amino acid sequences: type I (genotype I) and type II (genotypes II, III, IV and V). Positions are numbered according to the numbering of MnSOD nucleotide sequences deposited in the GenBank database. Polymorphic sites in the intron (positions 316–374) are highlighted in bold italic.

	3	51	54	87	93	129	132	144	189	195	201	208*	255	268	285*	300	322	326	330	334	349	351	352	355	358	360	368	370	386
I	T	T	G	A	C	T	G	T	G	T	G	T	T	T	A	T	A	C	A	A	T	G	G	G	A	–	G	T	
II	C	C	G	A	T	C	T	C	A	C	T	C	C	C	G	G	T	A	T	C	G	T	G	G	T	T	C	A	C
III	C	C	G	A	T	C	T	C	A	C	T	C	C	C	G	G	C	A	T	C	G	T	G	G	T	T	C	A	C
IV	C	C	A	A	T	T	T	C	A	T	T	C	C	T	G	G	T	G	T	C	G	T	A	G	A	T	C	A	T
V	T	C	G	T	C	C	T	T	G	T	T	C	C	T	G	G	C	G	A	A	G	A	G	A	G	G	–	A	T

*Non-synonymous mutations yielding amino acid mutation at positions 70 and 95 of the MnSOD protein sequence. Type I (genotype I sequences): Ser⁷⁰, Pro⁹⁵. Type II (genotype II, III, IV and V sequences): His⁷⁰, Gln⁹⁵.

subclade 1, isolates remained clustered with respect to host species. Most human TMC isolates were closer to the *T. interdigitale* neotype, in group A, which also contained four animal isolates from both natural hosts (two from guinea pig, close to the *T. interdigitale* neotype) and occasional hosts (one from a dog and one from a pig, closer to the zoophilic strain of *T. interdigitale* TMC-43). In group B, which was mainly zoophilic, TMC isolates from the same host clustered together, forming three distinct subgroups: chinchilla, mouse and human. The zoophilic strains of *T. interdigitale* (group B) were linked to *A. vanbreuseghemii*, confirming Takashio's mating-type studies, where some isolates derived from mice and chinchillas were shown to be able to mate with *A. vanbreuseghemii* (Takashio, 1973). Clade II zoophilic isolates were mostly derived from rabbits and guinea pigs, which are known to be natural hosts of the *Trichophyton* anamorph of *A. benhamiae*, whereas the natural host of *T. erinacei* is the hedgehog (Summerbell *et al.*, 2002; Weitzman & Summerbell, 1995). Analysis of animal TMC isolate distribution showed that only isolates from mouse and rabbit (natural hosts of the *T. mentagrophytes* species complex) clustered specifically within subclades 1 and 4, respectively. Other natural hosts (guinea pig and chinchilla) were represented in both subclades, but most chinchilla isolates (3/4) were closer to *T. interdigitale* (subclade 1) whereas most guinea pig ones (9/11) were closer to the *Trichophyton* anamorph of *A. benhamiae* (subclade 4). Occasional hosts (dog and pig) were also in both subclades. Taken as a whole, this distribution reflects the classical association of *A. vanbreuseghemii* with mice and chinchillas, and of *A. benhamiae* with rabbits and guinea pigs (Summerbell *et al.*, 2002), but it also highlights the complexity of the adaptation and evolution of the *T. mentagrophytes* species complex at the molecular level.

At the clinical level, there was a good correlation between the anamorph diversity based on morphology or clinical pattern, and the genotype. The morphology of subclade 1 human and animal TMC isolates was similar to the *T. interdigitale/A. vanbreuseghemii* phenotype (Table 1). Most colonies had a soft to downy texture with yellow-orange to brown reverse, associated with subspherical conidia, quite often spiral hyphae, and occasionally macroconidia. All human TMC isolates with *T. interdigitale* phenotypic features fell in this first clade, close to *T. interdigitale*, whereas TMC isolates closer to *T. erinacei* and *A. benhamiae* exhibited a different phenotype: mostly clavate or subspherical to ovoid microconidia, mycelium branchlets at right angles, no spiral hyphae (TMC-1, TMC-2, TMC-5 and TMC-9 in Table 1), and clustered with animal TMC isolates included in clade II (Fig. 1). Our results fitted well with the known predilection of dermatophyte species for certain body areas (De Hoog *et al.*, 1998; Fumeaux *et al.*, 2004; Graser *et al.*, 1998, 1999a, b; Kardjeva *et al.*, 2006; Ninet *et al.*, 2003; Summerbell *et al.*, 2002; Weitzman & Summerbell, 1995). Clade I human isolates (TMC-3, TMC-6, TMC-11, TMC-12, TMC-13 and TMC-14 in Table 1) were isolated from tinea pedis, like the neotype of *T.*

interdigitale, or from tinea unguium, whereas clade II human isolates (TMC-1, TMC-2, TMC-5 and TMC-9 in Table 1) were mostly associated with inflammatory lesions at body sites other than the feet, clustering with isolates from rabbits and guinea pigs which, as currently popular pets, might contribute to the increase of *A. benhamiae*-related *Trichophyton* strains isolated at present in France. A similar increase was recently reported in Japan and in Switzerland, where the introduction of unusual pets (in particular guinea pigs and rabbits) has been linked to the recent appearance of *A. benhamiae* (Fumeaux *et al.*, 2004; Kano *et al.*, 1998; Mochizuki *et al.*, 2001). Isolates TMC-4 and TMC-8 (group B, Fig. 1), which were isolated from inflammatory tinea corporis, were closer to rodent isolates (chinchilla or mouse), supporting the zoophilic origin of these *T. interdigitale* isolates. Within clade II, most isolates were derived from animals and clustered with the *Trichophyton* anamorph of *A. benhamiae*. Such taxa are generally encountered as zoophilic in France, Belgium and Spain, where they are expected to provoke acute inflammatory infections in human beings, but they can also be encountered as '*T. interdigitale*-like' in North America and Eastern Europe (Summerbell *et al.*, 2002). Here, the clinical features reported for our French human clade II isolates fit well with the zoophilic phenotype (Table 1) and confirm these data.

Conclusions

Phylogenetic analysis of French *T. mentagrophytes* complex isolates: contribution to a better understanding of the relationships between human and animal isolates.

This phylogenetic analysis of *T. mentagrophytes* complex included both human (14) and animal (27) isolates from a wide range of occasional and natural hosts. Our data are consistent with previous findings (De Hoog *et al.*, 1998; Faggi *et al.*, 2001; Graser *et al.*, 1999b; Kano *et al.*, 1998; Makimura *et al.*, 1998; Mochizuki *et al.*, 1990, 1996, 2001), confirming the close relationship between *T. interdigitale/A. vanbreuseghemii* and the *T. mentagrophytes* neotype, separate from *T. erinacei/A. benhamiae*, which seems to be primarily zoophilic (Graser *et al.*, 1999b; Kano *et al.*, 1998; Mochizuki *et al.*, 2001). Our TMC isolates clustered either with *T. interdigitale/A. vanbreuseghemii* or with the *Trichophyton* anamorph of *A. benhamiae*, but not with *T. mentagrophytes* neotype or *T. erinacei*. Mouse and rabbit isolates clustered specifically in clades I and II, respectively, but we did not find a systematic association between specific genotypes of *T. mentagrophytes* complex isolates and human or animal hosts, either natural (guinea pig or chinchilla) or occasional (dog or pig). This association had some morphological profile translations, since morphological features were mostly similar within isolates of the same clade. Microscopic features were more contributive than macroscopic ones, making them useful as indicators for routine morphological identification. They included the shape of the microconidia, previously described as a criterion to distinguish *A. vanbreuseghemii*- and *A. benhamiae*-related isolates (Graser *et al.*, 1999a). However, morphology was not always

characteristic and stable, and, therefore, not totally reliable, as previously described (Nenoff *et al.*, 2007). Thus, further molecular and morphological studies are needed.

In terms of evolution and host adaptation, our phylogenetic analysis supports the hypothesis of a recent divergence of *T. interdigitale*/A. *vanbreuseghemii*. As previously suggested (Makimura *et al.*, 1998; Mochizuki *et al.*, 1996), *T. interdigitale* could be the most anthropophilic *Trichophyton* species of this complex, representing a degenerate anamorph of A. *vanbreuseghemii*, having lost its mating ability and becoming a clonal population when it develops in the human host, like *T. rubrum* (Graser *et al.*, 2006).

The value of MnSOD and ITS for genotyping and phylogenetic analysis of the *T. mentagrophytes* complex. In terms of molecular approach, genotyping of dermatophyte strains has a number of potential epidemiological and clinical applications. As fungal species causing dermatophytosis can only be identified in 2–3 weeks after a positive culture, a rapid PCR method should be helpful in defining prevention and therapeutic strategies (Fernandez-Torres *et al.*, 2003; Kardjeva *et al.*, 2006; Mock *et al.*, 1998). The *T. mentagrophytes* MnSOD and ITS genes were targeted in order to improve the laboratory identification of dermatophytes. Although the results at the MnSOD locus confirmed the genetic heterogeneity of the *T. mentagrophytes* complex, they revealed a lower degree of diversity (similar to that of 28S rRNA: Ninet *et al.*, 2003) than the variable ITS locus. In particular, in clade I, all TMC isolates clustered in 'MnSOD genotype I', whereas five ITS genotypes could be differentiated for the same isolates. This probably results from the recent evolutionary dispersion of the anthropophilic dermatophytes, where some genes (e.g. coding for morphological or physiological characters) have rapidly fixed mutations, whereas housekeeping genes (such as MnSOD) have undergone relatively low fixation of mutations (Gupta *et al.*, 2002). However, the MnSOD gene clearly differentiated the four *T. mentagrophytes* complex species (*T. interdigitale*/A. *vanbreuseghemii*, *T. mentagrophytes*, *T. erinacei* and other A. *benhamiae* isolates) and *T. rubrum*, making it relevant as part of a multi-locus sequence typing approach (Devulder *et al.*, 2005).

On the other hand, comparison of the ITS region was suitable not only for phylogeny but also for species identification of dermatophytes, a critical requirement for applying appropriate treatment and preventive measures against dermatophytosis. An early determination of the anthropophilic or zoophilic character of the agent could lead to an earlier identification of the infection source (human, animal or soil), and therefore help in setting up suitable prevention and treatment measures.

Further, besides the current gold standard procedure based on direct microscopy of KOH preparations, combined with fungal culture, molecular identification of dermatophytes may help to identify fungal isolates that are not growing or that display unusual morphology. The recent development

of procedures to isolate DNA directly from clinical specimens (skin, hair or nails) would further enhance the potential of PCR in the clinical mycology laboratory, decreasing false-negative culture results (15%) and the misidentification (6%) of dermatophyte species (Kano *et al.*, 2003; Kardjeva *et al.*, 2006). In addition, contrary to usual belief, sequencing of the ITS locus is not much more expensive than identifying dermatophyte species by conventional methods (Kardjeva *et al.*, 2006). To summarize, molecular identification such as ITS sequencing could become part of the diagnostic gold standard for dermatophytosis, especially in reference laboratories with pre-existing molecular facilities.

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