Incidence of fungalysin and subtilisin virulence genes in dermatophytes

A. Lemsaddek, L. Chambel and **R. Tenreiro***

Universidade de Lisboa, Faculdade de Ciências, Centro de Biodiversidade, Genómica Integrativa e Funcional (BioFIG), Edificio ICAT, Campus da FCUL, Campo Grande, 1749-016 Lisboa, Portugal.
* Corresponding author: rptenreiro@fc.ul.pt

The dermatophytes are filamentous fungi that belong to the genera *Trichophyton, Epidermophyton* and *Microsporum* which can cause a variety of dermatophytosis common in animals and humans with possible transmission from animals to humans. The environment encountered by a dermatophyte during infection requires the release of specific enzymes to allow the penetration in host tissues.

Several proteases have already been considered potential virulence factors and in our study the presence of two families, one related with fungalysin (*MEP1-5* genes) and the other with subtilisin proteases (*SUB1-7* genes) were assayed by PCR amplification. For this purpose, newly developed specific primers were used to amplify these genes in a collection of 184 clinical and 21 environmental isolates and 28 reference strains, representing 14 species and/or varieties.

A high diversity of virulence gene profiles was observed (86 in 233 dermatophytes) and the presence of at least one gene was revealed in 91% (212/233) of the dermatophytes, with 44% (103/233) and 27% (64/233) being positive for the five *MEP* and seven *SUB* genes, respectively.

The presence/absence of each gene was related with taxonomic affiliation and origin of isolates, as well as patient gender and age and infection type. Except for *MEP3* and *SUB1* genes, several significant associations ($P<0.05$) and with odd ratios indicative of a higher relative risk of infection were found for the remaining *MEP* and *SUB* genes. Although a higher incidence was observed for anthropophilic species, the presence of several *MEP* and *SUB* genes in geophilic and zoophilic species turns all the dermatophytes potential agents of dermatophytosis. However, the absence of all *MEP* and *SUB* genes in some clinical isolates, as well as their occurrence in geophilic and zoophilic species, indicate that none of the screened genes is *per se* indispensable for the infection process.

Keywords: Dermatophytes; virulence factors; metalloprotease; subtilisin protease; *MEP* genes; *SUB* genes

1. Introduction

Dermatophytes are a group of keratinolytic fungi belonging to the anamorphic genera *Trichophyton*, *Microsporum* and *Epidermophyton* that are responsible for superficial mycoses common in human and animals. They are also capable of surviving without keratin but, in a superficial infection situation, the production and release of specific enzymes allows the fungus to penetrate the host's keratinized tissues [1-5]. Although keratin, elastin and collagen represent more than 25% of the mammal's weight [6], other proteins are present on the skin and may constitute substrates for the endo- and exoproteases produced by dermatophytes [7, 8]. Endoproteases are responsible for the cleavage of the protein and produce a high number of C or N terminus that enable the activity of exoproteases, being this synergistic cooperation necessary to provide short peptides and free aminoacids as nutrients for the dermatophytes.

Studies on dermatophyte pathophysiological mechanisms are scarce, but it is known that, like other filamentous fungi, the conidial germination can be divided in three steps: activation induced by environmental factors, isotropic growth and polarity growth [9]. Ultrastructure observation of human skin sections during dermatophyte infection shows a tenacious adherence between *Trichophyton mentagrophytes* spores and the *stratum corneum* after 12 hours. Invasion of the tissues is achieved by elongation of the germ tube that, by mechanical forces and secretion of various proteolytic and lipolytic exoenzymes, penetrates the cornified cells [10].

Several proteases have already been isolated from different species of dermatophytes and showed keratinolytic, elastinolytic and/or collagenolytic activities [3, 11, 12, 13]. In 2002, Brouta and co-workers [14] described a gene family (*MEP1*, *MEP2* and *MEP3*) coding for three endometalloproteases in *Microsporum canis* and reported the production of Mep2 and Mep3 proteases during *in vivo* experiences in guinea pigs. Mep3 was characterized and shows collagenolytic, elastinolytic and keratinolytic activities [15]. Other authors recognized two new genes, *MEP4* and *MEP5*, in *M. canis* as part of a five member gene family (named *MEP1-5*) encoding for secreted endometalloproteases (fungalysins), also identified in *Trichophyton rubrum* and *T. mentagrophytes* [16].

Another gene family (*SUB1*, *SUB2* and *SUB3*) encoding for serine proteases of the subtilisin family was reported in *M. canis* and the production of their respective proteases was observed during *in vivo* experiences in guinea pigs [17]. Later, seven genes (*SUB1-7*) coding for subtilisin proteases were isolated in *T. rubrum* by Jousson *et al*. [18]. The authors also reported the identification of orthologous genes in other dermatophyte species, three of them similar to the *SUB* genes of *M. canis*.

Dermatophytes are true pathogenic fungi infecting healthy individuals and the production of metalloproteases and subtilisin proteases during *in vivo* experiments is seen as an evidence of their importance in dermatophyte virulence [14, 17].

In the current study, the presence of putative virulence genes associated to dermatophyte isolates from diverse origins and species was assayed. Based on gene sequences of the metalloproteases and subtilisins available on GenBank database, 32 sets of primers for genes *MEP1* to *MEP5* and *SUB1* to *SUB7* were developed and used for PCR amplification in 184 human clinical isolates, 21 isolates recovered from swimming pool facilities and 28 reference strains from 14 species and/or varieties. The presence/absence of each gene was related with taxonomic affiliation and origin of isolates, as well as patient gender and age and infection type. The results were also compared with already reported dermatophyte secretomes.

2. Material and methods

2.1 Microorganisms

A total of 233 dermatophytes were included in the present study. Two hundred and five isolates were obtained from swimming pools (users and facilities) in Lisbon (Portugal) and healthcare centers in Lisbon-Portugal and Casablanca-Morocco and identified at species-level, based on macro- and microscopic morphology revealed by growth in specific media and using a molecular-based approach (data not shown). The isolates are members of the species *Epidermophyton floccosum* (2), *Microsporum canis* (28), *Microsporum audouinii* (23), *Microsporum gypseum* (6), *Trichophyton megninii* (1), *Trichophyton mentagrophytes* (80), *Trichophyton rubrum* (44), *Trichophyton soudanense* (2), *Trichophyton tonsurans* (12) and *Trichophyton violaceum* (7).

Twenty-eight dermatophytes obtained from Colección Española de Cultivos Tipo (CECT; Valencia; Spain) were also used as reference strains (CECT 2892^T; CECT 2893^T; CECT 2793; CECT 2795; CECT 2891; CECT 2894; CECT 2895; CECT 2896; CECT 2897; CECT 2898; CECT 2921; CECT 2989; CECT 20116; CECT 2908; CECT 2990; CECT 2998;CECT 2999; CECT 2902; CECT 2899; CECT 2900; CECT 2796; CECT 2991; CECT 2956^T; CECT 2957; CECT 2958; CECT 2959; CECT 2794; CECT 2992).

2.2 DNA isolation and PCR screening for *MEP* and *SUB* genes

Dermatophytes were cultured on Sabouraud Dextrose Agar for 7-15 days at 28ºC and after growth the mycelium was recovered and frozen at -80ºC. The mycelium was finely grounded with a pestle in a mortar using liquid nitrogen and total DNA was extracted by the guanidium thiocyanate method adapted from Pitcher *et al*. [19].

The oligonucleotide primers designed and used in the present study are listed in Table 1 and were purchased from Invitrogen (Paisley, UK). Primers were originally developed based on nucleotide sequences of the genes *MEP1-5* and *SUB1-7* available on GenBank database for the species *M. canis, T. rubrum* and *Arthroderma benhamiae*.

All PCR amplifications were performed in a thermo RoboCycler (Stratagene), using a total volume of 50 µl and including: 1x reaction buffer, 0.2 mM of each deoxynucleoside triphosphates, 2 mM $MgCl_2$, 0.5 mM of each primer, 1 U of *Taq* DNA polymerase and 100 ng of dermatophyte DNA. All reagents were purchased from Invitrogen. PCR cycling conditions consisted of: 94ºC for 3 min followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC and 1 min at 72ºC and a final extension step of 5 min at 72ºC. The PCR products (10 µl) were resolved by agarose gel electrophoresis (1% w/v) in 0.5x TBE buffer (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA; Invitrogen) at 90 V for 1 h. DNA was visualized by UV light after staining with ethidium bromide and photographed with KODAK 1D image analysis software (version 3.5.2).

Table 1 Primers used for PCR amplification of *MEP* and *SUB* genes and expected size of amplicons

Gene		*Microsporum canis*		*Trichophyton rubrum*		*Arthroderma benhamiae*	
		Primers (5´---> 3´)	Size (bp)	Primers (5´---> 3´)	Size (bp)	Primers (5´---> 3´)	Size (bp)
MEP1	For[a]	ATGCACGGCCTCTTGCTTG		GCCACTGAGCTGGTTAAG		GCTGTCGACCTGAATGAG	
	Rev	CTTGCACTCCTTTGGAACCTCG	2200	CTTTGGATCGAACTTAGC	1650	CAAGATACTTGCCGTCATTTG	1800
MEP2	For	CTTTGGGCGTTGCTGGTCAC		AGAGTTCCTGACTCGGAC		TACTCCAAGGATGACGAC	
	Rev	TGTCGAAGTTGTTGGTGTAGCC	1900	ACTCGTGGATGACAATACC	1400	TCGATCAAGTTCCACAGG	1501
MEP3	For	CTGCCCATGAATGTGCTCG		GCCATGTCCTTCTCCAAG		TGAAGGCCAAGTACATGG	
	Rev	AGCCTGGTGGAAGTTGGTTGC	2300	AGACCACGCTTAGCAAAG	2000	TTGGTGGAGTAGAGGTAGG	1650
MEP4	For	TGATACCAGCGCCTACTTC		ATCGTGATTCCTTTAGCACC		AGAGCGATGCTGTTCCTAAG	
	Rev	TTAAGGATGGTAAAGTCATTGC	1200	TCGCCCATGGTATAGTCAG	2000	GAAGCCCAAATAGTACCAATG	1650
MEP5	For	CGAGCTGTTGATCTGAATG		CCAGCTACATGAGTTCAGATG		GTGTAGACCTGGATGCCTATC	
	Rev	TTGAGGCTGGTGGAATAG	1650	ACAGGATGTGTAGACCAATGG	1650	ATCGGCATCGATAATGGC	2000
SUB1	For	TATCTCTCTTGCGGCTGTGTCC		ATCCTGTCTATGCCTCATG		TATCTTCGACGAAGAGACC	
	Rev	GTTCCAGAATCTGCCGAAGGTC	1540	AATCGAAGTCGAAGTTATC	1500	GGGCAGAGATAATGTTGG	850
SUB2	For	TGAACCTCGGCCTGCTTCTC		ATATCTCGTCACACTGAAGG		ACTTTGGCTTGCTTCTTCTC	
	Rev	TTCTTGGGCAGTGTGAACTTGC	1496	CCTGGATGCCATTGTACAC	1400	GCATGACTTGATGAGTTCTC	1300
SUB3	For	TGGGCTGCATCAAGGTTATCTC		TTATCTCCGTCTTCCTAGC		TATCCTCAAAGATGACAAGG	
	Rev	CCGTTGTAGAGGAGCTTGTTGG	1388	AGCAACGCTAACACCCTG	1300	AGCACCAACACCACAAAC	1000
SUB4	For	-	-	AAGACTCAGGGCCACAAG		GACTCAGGGCCACAAGAAC	
	Rev	-	-	TTCCGATCATGTAGGCAC	1200	CATGTAGGCACCCATACC	1200
SUB5	For	-	-	GAAGTTGTGCCCAATGGC		TGAAGTCGTGCCCAATGG	
	Rev	-	-	CTCCAGGCCTAGCAGAAAG	1100	GTCCAGGATTGCGGATAG	1250
SUB6	For	-	-	CGATTCAGAACTGATTATGATG		GCGTTGTCAACTGTCAATG	
	Rev	-	-	GAGGTTTGGAGGCAGGTTC	1650	GGCTTAAGGGAGGTGATATC	1200
SUB7	For	-	-	CTTGCCTAGACACTTCAATG		GCTGAGATCATGGAGACTC	
	Rev	-	-	ATGAGGTAGGCACCGAGAC	1384	GGAGCGTAGACATCAACTG	1100

[a] For: forward primer; Rev: reverse primer.

2.3 Data analysis

The chi-square analysis of contingency tables was used to test the statistical independence between occurrence of virulence factors and genus allocation of isolates (*i.e. Microsporum* and *Trichophyton*), origin of isolates (swimming pool or human infection), patient age (young, up to 18 years; adults, 19 to 60 years; and seniors, over 60 years), patient gender and infection type (*tinea barbae*/*faciei*, *tinea capitis*, *tinea corporis*, *tinea cruris*, *tinea manuum*, *tinea pedis* and *tinea unguium*) [20]. Whenever appropriate, odd ratios (ORs) and 95% confidence intervals (CIs) were also calculated [21].

3. Results

In the present investigation, a screening for the presence of 12 virulence genes (five *MEP* genes and seven *SUB* genes) was carried out on a total of 233 dermatophytes, representing 14 *taxa*. Regarding the species for which no *MEP* and *SUB* genes were described, the screening was performed using all primers developed from *M. canis*, *T. rubrum* and *A. benhamiae* nucleotide sequences. Concurrently and because *SUB4-7* genes are not yet described for *M. canis*, these genes were screened based on primers developed for *A. benhamiae* and *T. rubrum*.

Among the fungi under analysis, 19% (45/233) showed to harbor the 12 genes, the majority being members of *T. mentagrophytes sensu lato* (*i.e.* all the varieties and both anamorphs and teleomorphs of *T. mentagrophytes*) and included swimming pool isolates (users and facilities) and healthcare isolates from Morocco and Portugal. The 12 genes were also successfully amplified for the reference strains of *A. benhamiae* (CECT 2793, CECT 2891, CECT 2892^T, CECT 2894, CECT 2895, CECT 2896, CECT 2897, CECT 2898 and CECT 2921), *Arthroderma vanbreuseghemii* (CECT 2902) and *T. rubrum* (CECT 2959).

Concurrently, none of the 12 genes could be amplified for 9% (21/233) of the isolates obtained from the swimming pool (users and facilities) and a healthcare in Lisbon, including isolates belonging to the species *T. mentagrophytes sensu lato* (14 isolates), *T. rubrum* (4 isolates) and *M. gypseum* (1 isolate), as well as the reference strains *A. vanbreuseghemii* (CECT 2900) and *T. mentagrophytes* var. *interdigitale* (CECT 2958). Since part of the 18S rRNA region, described by Bock *et al.* [22] as present in all dermatophytes species, was successfully amplified in all the isolates, these negative results were not due to PCR inhibition.

3.1 Metalloprotease genes

Among the analyzed isolates, 44% (103 of 233) were positive for the five *MEP* genes, belonging to the species *Epidermophyton floccosum*, *Microsporum audouinii*, *M. canis*, *T. mentagrophytes sensu lato* and *Trichophyton tonsurans*, as well as the reference strains of *A. benhamiae* (CECT 2793, CECT 2891, CECT 2892^T, CECT 2894, CECT 2895, CECT 2896, CECT 2897, CECT 2898 and CECT 2921), *A. vanbreuseghemii* (CECT 2902) and *T. rubrum* (CECT 2959). The occurrence of *MEP* genes amongst dermatophytic species is presented in Fig. 1.

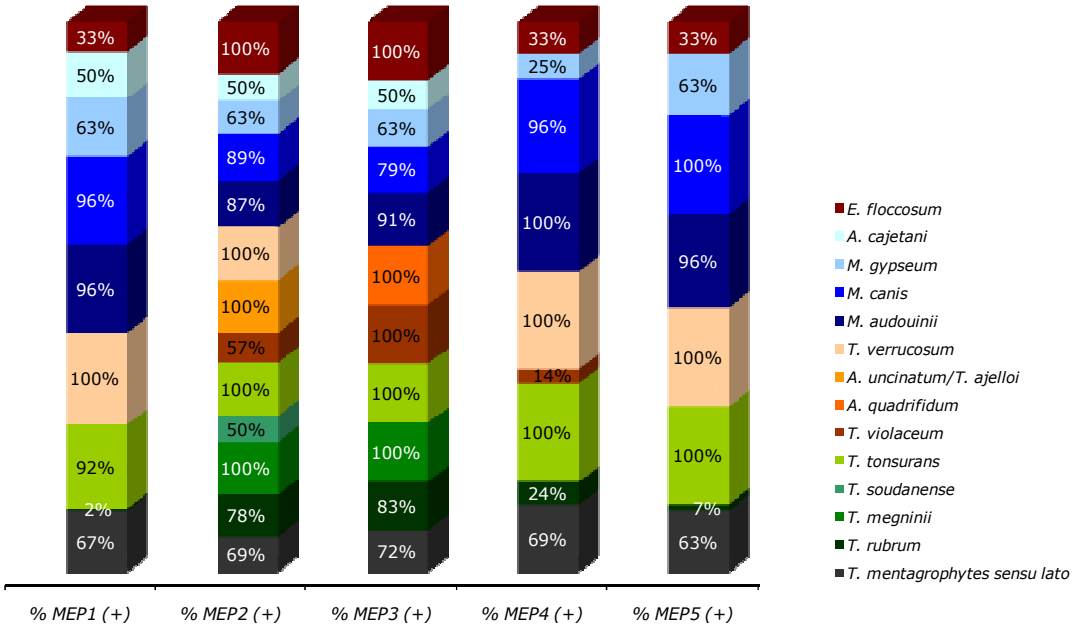

Fig. 1 Occurrence of *MEP* genes on the analyzed species of dermatophytes.

Simultaneously, 13% (30/233) of the isolates, belonging to the species *M. gypseum*, *T. mentagrophytes sensu lato*, *T. rubrum* and *T. soudanense*, and the reference strains *T. mentagrophytes* var. *erinacei* (CECT 2956^T), *T. mentagrophytes* var. *goetzii* (CECT 2957), *T. mentagrophytes* var. *interdigitale* (CECT 2958) and *A. vanbreuseghemii* (CECT 2900), seem to lack these five genes.

The results also showed that 58% (134/233) of the isolates harbor the *MEP1* gene and statistical analysis revealed significant associations between *MEP1* presence and the genus *Microsporum* (P<0.05), the isolates recovered from females (P<0.05), the isolates obtained from young patients (P<0.05) and the isolates responsible for *tinea capitis* and *tinea corporis* infection types (P<0.05). To measure the risk associated with the presence of *MEP1* gene, odd ratios and 95% confidence intervals (CI) were calculated and showed a significant relative risk that was higher in *tinea corporis* (OR=4.45; 95% CI, 1.51 to 13.10) and *tinea capitis* (OR=5.76; 95% CI, 2.30 to 14.42) comparatively to *tinea pedis*.

MEP2 was detected in 76% (178/233) of the analyzed isolates independently of the fungus genus or the age of the patients from which the isolates were obtained, while its presence seems to be related with isolates obtained from female patients (P<0.05), recovered from human infections (P<0.05) and causing *tinea barbae/faciei*, *tinea capitis*, *tinea corporis*, *tinea manuum* and *tinea cruris* (P<0.05). The relative risk related with the presence of *MEP2* seems to be higher for isolates obtained from human infection relatively to those obtained from the swimming pool (OR=5.36; 95% CI, 1.60 to 17.91) and in *tinea corporis* (OR=11.94; 95% CI, 1.49 a 95.63) or *tinea capitis* (OR=3.26; 95% CI, 1.16 to 9.11) comparatively to *tinea pedis*.

MEP3 was successfully amplified for 78% (181/233) of the isolates with no statistically significant differences observed for any of the comparisons performed.

Amplification of *MEP4* was positive for 62% (145/233) of the isolates, being significantly associated with *Microsporum* isolates (P<0.05), those obtained from young and adult patients (P<0.05) and responsible for *tinea barbea/facei*, *tinea pedis*, *tinea unguium* and *tinea cruris* (P<0.05). Simultaneously, the relative risk associated with the presence of *MEP4* seems to be higher in isolates obtained from young patients relatively to those obtained from adult (OR=6.61; 95% CI, 2.67 to 16.33) and senior (OR=6.80; 95% CI, 2.38 to 19.44) patients, as well as for isolates recovered from adults comparatively to seniors (OR=2.55; 95% CI, 1.08 to 6.04). The presence of *MEP4* seems to raise the risk of developing *tinea corporis* (OR=3.88; 95% CI, 1.25 to 11.94) and *tinea capitis* (OR=5.38; 95% CI, 2.03 to 14.22) infections comparing to *tinea pedis*.

MEP5 gene was detected in 57% (133/233) of the isolates being significantly associated with *Microsporum* isolates (P<0.05) and with those recovered from young patients (P<0.05). Concurrently, the risk related with the presence of *MEP5* seems higher in isolates recovered from young patients relatively to adult (OR=4.53; 95% CI, 2.12 to 9.68) and senior (OR=5.39; 95% CI, 2.11 to 13.73) patients.

3.2 Subtilisin genes

Screening for the presence of *SUB* genes revealed that a group of isolates, representing 27% (64/233), were positive for the seven *SUB* genes, while in 14% (33/233) none of the *SUB* genes was observed. The occurrence of *SUB* genes amongst dermatophytic species is presented in Fig. 2.

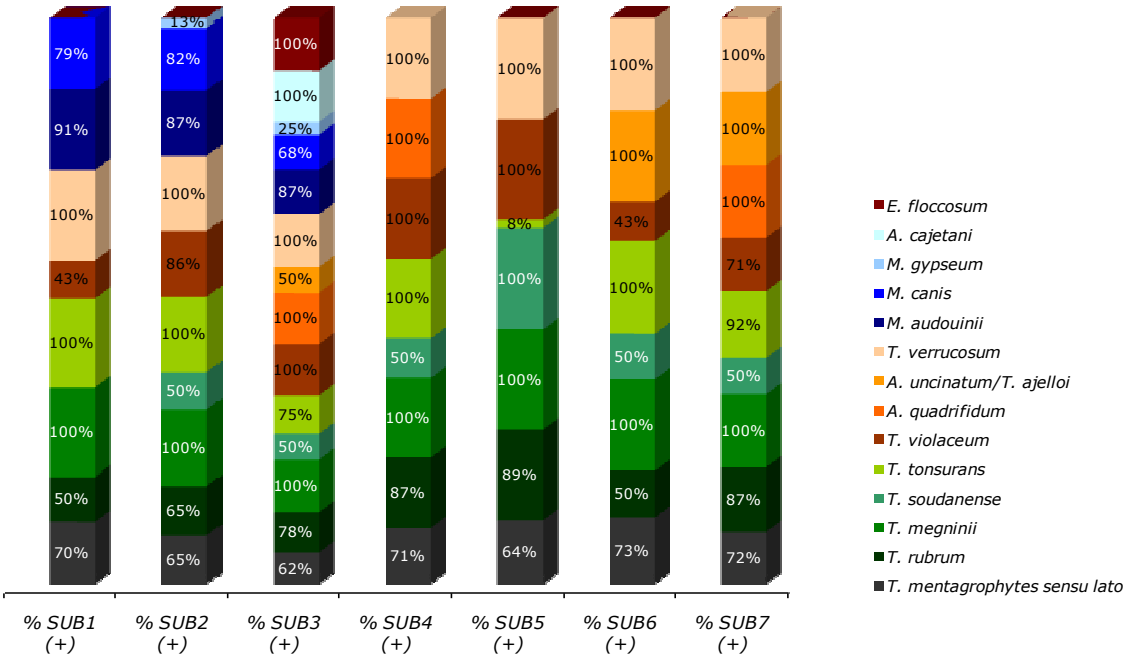

Fig. 2 Occurrence of *SUB* genes on the analyzed species of dermatophytes.

SUB1 gene was detected in 65% of the isolates (151/233) from eight species and, with the exception of *T. soudanense* and *E. floccosum*, this gene was found in all anthropophilic and zoophilic species, while no geophilic isolates seem to possess this gene. For *SUB1* no statistically significant associations were found regarding genus allocation, patient age and gender or infection type.

Concerning *SUB2* gene, a positive amplification was obtained for 68% (158/233) of the dermatophytes under analysis, being significantly associated and raising the risk in isolates obtained from infected patient comparatively to those obtained from the swimming pool ($P<0.05$; OR=5.19; 95% CI, 1.49 to 18.05).

SUB3 gene was detected in a high percentage (70%; 163/233) of the isolates with a significant association being observed for isolates recovered from female patients ($P<0.05$) and human infections ($P<0.05$) and responsible for *tinea capitis*, *tinea corporis* and *tinea manuum* ($P<0.05$). The relative risk associated with the presence of *SUB3* seems to be higher in isolates obtained from human infections (OR=3.74; 95% CI, 1.13 to 12.38), *tinea corporis* (OR=3.53; 95% CI, 1.05 to 11.76) and *tinea capitis* (OR=5.49; 95% CI, 1.86 to 16.14) comparatively to *tinea pedis*.

The serine proteases genes *SUB4-7* were detected only in species belonging to the genus *Trichophyton*, with intra-genus frequencies of 78% (132/169) for *SUB4* and *SUB7* and 68% for *SUB5* (115/169) and *SUB6* (114/169). All these four genes are significantly associated with isolates recovered from adult and senior patients ($P<0.05$) and a significant association was observed between *SUB4* and *SUB7* genes and *tinea barbae/faciei*, *tinea manuum*, *tinea pedis*, *tinea unguium*, and *tinea cruris* ($P<0.05$). As for *SUB5* and *SUB6*, a significant association was also observed between their presence and isolates causing *tinea manuum*, *tinea pedis*, *tinea unguium* and *tinea cruris* ($P<0.05$), while *SUB5* is significantly associated with isolates obtained from human infections ($P<0.05$).

Concerning the relative risk related with the presence of *SUB4-7* genes, it seems raised for *tinea unguium* relatively to *tinea corporis* (*SUB4* - OR=5.2; 95% CI, 1.44 to 18.71; *SUB5* - OR=4.11; 95% CI, 1.19 to 14.13; *SUB6* - OR=5.03; 95% CI, 1.46 to 17.37; *SUB7* - OR=6.22; 95% CI, 1.71 to 22.58) and for *tinea unguium* comparatively with *tinea capitis* (*SUB4* - OR=10; 95% CI, 3.05 to 32.76; *SUB5* - OR=10.13; 95% CI, 3.17 to 32.33; *SUB6* - OR=5.68; 95% CI, 1.92 to 16.78; *SUB7* - OR=12.8; 95% CI, 3.81 to 42.93). The risk associated with *SUB4* seems higher in isolates recovered from *tinea unguium* relatively to those obtained from *tinea pedis* (OR=3.2; 95% IC, 1.04 to 9.78) and *SUB5* seems to raise the risk to develop infection, since an OR=11.13 (95% CI, 1.4 to 88.14) was found when human infection isolates were compared with swimming pool ones.

3. Discussion

At least one *MEP* gene was detected for isolates from different origins and all the analyzed species showed to harbor one or more metalloprotease coding genes. For *MEP1*, the results obtained showed a high incidence of this gene among our collection of isolates, pointing towards its role in the infection, probably in the early stages of the process. In fact, Brouta *et al.* [14] did not detect the Mep1 protein on days 14 and 21 postinoculation, during *in vivo* experiences with guinea pigs. Considering our results, *T. rubrum,* which is responsible for a high number of dermatophyte infections in Europe and USA [23] and considered well adapted to humans, seems to lack *MEP1* gene (except strain CECT 2959). This absence could be related with an "escape mechanism" to the human immunologic system.

Concerning the *MEP2* gene, its detection was possible for 13 of the 14 analyzed species, being the only *MEP* gene detected in one isolate of *T. soudanense* (responsible for *tinea capitis*) and in the geophilic species *A. uncinatum/T. ajelloi*. These results, associated with the fact that Mep2 protease has been detected during *in vivo* experiences [14], point to an important role of this protein during the infection process. In their study, Jousson *et al.* [16] did not find Mep2 protease after inoculating *T. rubrum*, *T. mentagrophytes* and *M. canis* in a medium containing soy as sole nitrogen source. Distinct results were obtained by Giddey *et al*. [24], since these authors detected the production of Mep2 protein after growth of *T. violaceum* in the same medium. Another investigation [25] detected the *MEP2* mRNA after growth of *A. gypseum* in a hair medium, whereas this mRNA was not found after growth in Sabouraud Dextrose Agar, confirming that *MEP2* expression was induced by the presence of hair constituents. However, Mep2 is still not fully characterized and its exact role during dermatophyte infection remains to be determined.

The high incidence of *MEP3* gene among our collection of isolates seems to indicate an important role of its coding protein in the infection process. In fact, Mep3 has already been characterized and showed elastinolytic, keratinolytic and collagenolytic activities [15], being also one of the most secreted proteins during growth on soy medium, and turning evident its importance during infection. *T. soudanense*, *A. uncinatum/T. ajelloi* and *T. verrucosum*, represented by a small number of isolates, are the three species where *MEP3* could not be detected. Knowing the proteolytic activity of Mep3, the effective absence of its coding gene on these species has to be proven by the analysis of more isolates. In case of confirmation, it can be hypothesized its replacement by another gene, coding for a protein with similar activities, especially regarding Mep3 keratinolytic activity which will allow the fungus to degrade one of the most important constituent of the skin, nails and hair.

The presence of *MEP4* was detected in more than half of the analyzed isolates. The protein encoded by *MEP4*, along with Mep3, was the most secreted by *M. canis*, *T. mentagrophytes* and *T. rubrum* on soy medium [16]. Using the same medium, Giddey *et al*. [24] detected these proteases on the supernatant of *A. vanbreuseghemii*, *T. equinum*, *T.*

tonsurans, *T. rubrum*, *T. soudanense* and *T. violaceum*. mRNAs corresponding to *MEP3* and *MEP4* were also detected on keratin medium inoculated with *T. rubrum* [26].

Since only *T. soudanense* and *A. uncinatum*/*T. ajelloi* (both represented by two isolates) lack *MEP3* and *MEP4* genes, our results are in agreement with the reported above by other authors. For the majority of the isolates belonging to other species, at least one of these two genes was detected pointing towards an important role of their corresponding proteases during infection.

Concerning *MEP5* gene, even if it was detected only in eight species, the high percentages observed suggest an important role of its coding protease. Simultaneously, the species in which *MEP5* was not detected are, with the exception of *T. rubrum*, poorly represented in the analyzed collection. As for *MEP1*, the low detection of *MEP5* in *T. rubrum* isolates could be related with an "escape mechanism" to the immunological system. However, a future study including an additional number of isolates, representing all species and the three ecological groups, will help to unravel the putative role of Mep5 protein during the infectious process.

In the analyzed dermatophyte species at least one *SUB* gene was detected and all of them possess *SUB3*, which codes for a protease showing activity towards albumin, collagen, keratin and elastin. The species *T. mentagrophytes sensu lato*, *T. rubrum*, *T. megninii*, *T. tonsurans*, *T. violaceum* and *T. verrucosum* harbor the seven *SUB* genes. *T. soudanense* only lacks *SUB1* while *SUB2*, *SUB5* and *SUB6* were not detected for *A. quadrifidum*. Concerning *A. uncinatum/T. ajelloi*, *SUB3*, *SUB6* and *SUB7* were detected whereas only *SUB1-3* was successfully amplified for *M. canis* and *M. audouinii*. Two genes were observed for *M. gypseum* (*SUB2-3*), while *A. cajetani* and *E. floccosum* only harbor *SUB3*. *SUB1* gene was detected in all the analyzed anthropophilic species, except *T. soudanense* and *E. floccosum*, while none of the geophilic isolates included in this investigation seem to harbor this gene pointing towards a high importance of Sub1 during the infection process. Indeed, Baldo *et al*. [27] observed that Sub1 protease was produced by *M. canis* arthroconidia during the early stages of infection, postulating that it may be related with adherence to and/or invasion of corneocytes.

SUB2 gene was successfully amplified in 10 of the analyzed species, being *E. floccosum* the unique anthropophilic species where it was not detected. Although Sub2 protein is not yet characterized, its detection during *in vivo* experiments [17] and the fact that 13% of the analyzed *M. gypseum* isolates harbor its coding gene indicate that this protein may be very important during the infection process.

The Sub3 protein was detected in a culture medium containing soy protein as sole nitrogen source and shows activity towards albumin, collagen, keratin and a reduced activity on elastin [18]. The detection of *SUB3* gene in a high percentage of the analyzed isolates, representing all genera and species, may explain the dermatophytes' ability to degrade keratin and certainly contributes to their capacity to cause infections in humans and animals. Furthermore, Sub3 was shown to be involved in adherence of arthroconidia to corneocytes [27]. However, Sub3 inhibition failed to totally hinder arthroconidia adherence pointing to the presence of other molecules also involved during this process.

The *SUB4-7* genes could not be detected in any of the isolates of the genera *Microsporum* and *Epidermophyton*. Of the proteins encoded by these genes, only Sub4 is well characterized and has activity towards albumin, collegen, keratin and elastin. Although Sub4 was detected on soy medium inoculated with *T. rubrum* and *T. mentagrophytes* [18], the results of the present study point towards its reduced role during the infection process as neither the anthropophilic nor the zoophilic or geophilic species of *Microsporum* possess the *SUB4* gene. Sub5 and Sub6 proteins showed no activity towards collagen, keratin, albumin and casein and were not produced on soy agar by any of the three species studied by Jousson *et al*. [18, 28]. However, the proteins may have activity towards another component of the skin which may give advantage to the isolates harboring its corresponding genes. Concerning the protein coded by *SUB7*, its expression was only detected on soy medium inoculated with *A. benhamiae*. The results of the present study point to a reduced role of Sub7 protein during the infection process. Although *T. mentagrophytes sensu lato* and zoophilic and anthropophilic species of *Trichophyton* harbor this gene, the fact that *SUB4-7* genes were not detected in isolates of *Microsporum* spp. and *E. floccosum* (and thus not interfering with their capacity to cause infections) may indicate a minor role of the corresponding proteins during infection.

Although represented by different number of strains, anthropophilic, zoophilic and geophilic species were included in our study and all possess at least one of the investigated genes, being the presence of more virulence determinants related with the anthropophilic and zoophilic species. However, the presence of genes coding for proteases with activities against keratin on geophilic species give them the ability to degrade keratin in soil (animal hair), being also able to cause infections in human (*e.g. M. gypseum*).

The presence of *MEP* and *SUB* genes in geophilic and zoophilic species turns all the dermatophytes potential agents of dermatophytosis. However, the absence of all *MEP* and *SUB* genes in some clinical isolates, as well as the occurrence of these genes in geophilic and zoophilic species, indicates that none of the screened genes is indispensable for the infection process. Then, the proteases coded by *MEP* and *SUB* genes may be related with the severity of the infection rather than the ability to cause infection, being also possible that other infection-related factors remain to be discovered.

The characterization of Mep and Sub proteins remains crucial to understand the real involvement of these proteins during the infection process and will define if the presence of these gene families constitutes (or not) an advantage for dermatophytes.

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