UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

INTEIN PRP8 EM FUNGOS DERMATÓFITOS: IDENTIFICAÇÃO MOLECULAR E ASPECTOS EVOLUTIVOS.

HANS GARCIA GARCES

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia de Parasitas e Microrganismos.*

Orientador: Prof. Dr. Eduardo Bagagli

Botucatu - SP
2017

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Palavras-chave: Dermatófitos; Filogenia; Identificação molecular; Intein PRP8.

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¨ Al mundo nuevo corresponde la Universidad Nueva. A nuevas ciencias que todo lo invaden, reforman y minan nuevas cátedras. Es criminal el divorcio entre la educación que recibe una época y la época… En tiempos teológicos, universidad teológica. En tiempos científicos, universidad científica. ¨

José Martí (1853-1895)

Resumo.

Os dermatófitos são um grupo de fungos constituídos pelos gêneros *Trichophyton*, *Epidermophyton* e *Microsporum* que têm a habilidade de degradar a queratina. É por essa razão que podem colonizar a pele do homem e dos animais, embora também possam crescer no ambiente, geralmente em solos com restos de queratina. As características morfológicas permitem a identificação e classificação taxonômica das espécies, mas realizar um diagnóstico baseado nestas características pode levar a erros. As técnicas moleculares ajudam a realizar diagnósticos mais rápidos baseados em uma identificação molecular e também têm possibilitado importantes avanços quanto aos estudos filogenéticos, sendo as sequências ITS1-5.8S-ITS2 as mais utilizadas. No entanto, estes marcadores não são suficientes para identificar e elucidar todas as relações filogenéticas e taxonômicas dos dermatófitos devido à ampla variedade de espécies existentes, -sendo necessário novos marcadores genéticos, como o intein PRP8. Os inteins são elementos genéticos parasitas, de natureza proteica, presentes em alguns fungos e estão associados a importantes genes altamente conservados. O intein PRP8 está localizado no gene *PRP8* que codifica para a proteína PRP8 associada ao complexo do spliciosoma. O presente estudo visa caracterizar o intein PRP8 em fungos dermatófitos, de forma a empregar estes elementos genéticos como marcadores moleculares para uma correta identificação destas espécies e elucidações das relações filogenéticas do grupo. Para realizar este objetivo, foram caracterizadas molecularmente 45 cepas usando as regiões nucleares ribossomais ITS1-5.8S-ITS2 e D1/D2 e posteriormente a região do intein PRP8 de 40 cepas foi sequenciada para determinar as características do intein. A presença do intein foi comprovada em todas as espécies avaliadas, apresentando um full-intein com uma Homing Endonuclease presumivelmente ativa. As construções filogenéticas obtidas usando as sequências do intein são correspondentes as construídas a partir de genes nucleares ribossomais, demonstrando a validade do intein para ser incluído em futuros estudos filogenético associados a taxonomia e determinação de espécies. O polimorfismo na região da Homing Endonuclease permitiu a identificação de algumas espécies de *Microsporum* mediante um ensaio de PCR-Eletroforese.

Palavras chaves: Dermatófitos, Intein PRP8, Identificação molecular, filogenia.

Abstract

Dermatophytes are a fungal group composed by the genera *Trichophyton*, *Epidermophyton* and *Microsporum* with the ability to degrade keratin. That is why they can colonize the skin of man and animals, although they can also grow in the environment, usually in soils with remains of keratin. The morphological characteristics allow the identification and taxonomic classification of the species, but a diagnosis based on these characteristics is difficult. Molecular techniques made diagnoses based on molecular identification faster and made possible important advances in phylogenetic studies, with ITS1-5.8s-ITS2 sequences being the most used. However, these markers are not enough to elucidate all the phylogenetic relationships and taxonomic of dermatophytes due to the wide variety of existing species, and new genetic markers may be needed for correct identification and phylogenetic studies, such as the PRP8 intein. Inteins are parasitic genetic elements of a protein nature present in some fungi and are associated with important highly conserved genes. The PRP8 intein is located within the *PRP8* gene coding for the PRP8 protein associated with the spliciosome complex. The present study aims to characterize the PRP8 intein in dermatophyte fungi, in order to use these genetic elements as molecular markers for a correct identification of these species and elucidations of the phylogenetic relationships of the group. To accomplish this goal, 45 strains were molecularly characterized using the ITS1-5.8S-ITS2 and D1 / D2 ribosomal nuclear regions and subsequently the PRP8 intein region of 40 strains was sequenced to determine the intein characteristics. The presence of intein was confirmed in all evaluated species, presenting a full-intein with a presumably active Homing Endonuclease. The phylogenetic construction obtained using the intein sequences correspond to those constructed from ribosomal nuclear genes, demonstrating the intein validity to be included in future phylogenetic studies associated with taxonomy and species determination. Polymorphism in the Homing Endonuclease region allowed the identification of some *Microsporum* species by a PCR-Electrophoresis assay.

Key words: Dermatophytes, PRP8 intein, molecular identification, phylogeny.

Lista de abreviaturas:

BLAST: Basic Local Alignment Search Tool (Inglês).

BLASTn: BLAST nucleotide (Identificaçao baseada nas sequencias de DNA).

BLASTp: BLAST protein (Identificaçao baseada nas sequencias de proteínas).

CBS: Centraal bureau voor schimmelcultures (Centro de estudos na área da Micologia, localizado na Holanda).

cDNA: DNA codificante.

CHSI: Chitin Synthase Class I.

D1/D2: Região variável da subunidade maior 28S do rRNA.

dS/dN rate: radio de substituições sinônimas e não- sinônimas.

HE: Homing endonuclease (Inglês).

IGS: Intergenic spacers (espaçadores intergênicos).

ITS: Internal Transcribed Spacer. (Presente na região nuclear ribossomal).

LSU: Large subunit (subunidade maior). Referido ao rRNA.

mDNA: DNA mitocondrial.

mRNA: RNA mensageiro.

ML: Maximum Likelihooh method.

NCBI: National Center for Biotechnology Information (Inglês).

PRP8: Proteína central do spliceosomo.

PCR: Polimerase Chain Reaction (Inglês).

rDNA: Ribossomal DNA (rRNA ao nível gênico).

RFLP: Restriction Fragment Length Polimosphism (Inglês).

rRNA: Ácido ribonucleico ribossomal.

SSUrDNA: Small Subunit ribosomal DNA (sequência de DNA que codifica para a subunidade menor do rRNA).

ThrRS: RNA de transferência para o aminoácido treonil (referido como Intein ThrRS).

TOP2: Topoisomerase tipo II.

tRNA: RNA de transferência.

VMA: ATPase da membrana vacuolar (Referido como Intein VMA).

WGS: Whole Genome Shotgun (Inglês).

Índice

1. Introdução

Os dermatófitos.

Os dermatófitos são ascomicetos da ordem Onygenales, família Arthrodermataceae que pertencem aos gêneros do estágio anamorfo (assexual ou imperfeito) *Trichophyton*, *Microsporum* e *Epidermophyton*. Estes organismos têm a capacidade de invadir tecidos queratinizados (pele, pelos e unhas) resultando numa doença chamada dermatofitose ou tinea. Visando sua ecologia, podem ser classificados como espécies antropofílicas (espécies que afetam primariamente ao homem), zoofílicas (espécies que afetam primariamente os animais) e geofílicas (espécies que costumam ter vida livre, sendo moradores do solo). Os gêneros *Trichophyton* e *Microsporum* são compostos por várias espécies, que podem ser geofílicas, zoofílicas e antropofílicas. Já o gênero *Epidermophyton,* contém uma única espécie (*E. floccosum*) que só afeta os humanos. Os membros dos gêneros *Trichophyton* e *Microsporum* costumam infectar pêlo, pele e unhas e *E. flocossum* só infecta pele e unhas, não invadindo o cabelo. Em pacientes imunocomprometidos, as infecções por dermatófitos podem ser formas severas e insidiosas se manifestando como granulomas dermatofíticos e kerion (Rippon, 1988; Da Silva Lacaz *et al.*, 2002; Jung *et al.*, 2009; Lourdes *et al.*, 2014; John *et al.*, 2016; Hay, 2017; Trocoli Drakensjo *et al.*, 2017)

A similaridade dos dermatófitos consiste em seu requerimento nutricional, pois todos eles têm a capacidade de digerir a queratina. A queratina é uma proteína altamente insolúvel e seu uso como substrato é raro na natureza. Ela forma parte da capa protetora externa dos répteis, aves e mamíferos em estruturas como o estrato córneo, garras, bicos, unhas e penas (Rippon, 1988). Sua natureza queratinofílica sugere que estes fungos foram originados quando a vida chegou na terra desde a água porque os vertebrados aquáticos (ex. baleias) carecem de anexos que apresentem queratina. Não existe nenhum dermatófito que seja aquático, sendo inibidos por altas concentrações de sal (Sharma e Gräser, 2011).

Algumas espécies são cosmopolitas (Nweze e Eke, 2016; Hay, 2017; Zhan e Liu, 2017), no entanto, outras possuem limites geográficos bem estabelecidos como ocorre com *T. soudanense* (espécie classificada como *T.rubrum* de população africana segundo Gräser et al. 2008) encontrado na África e *T. concentricum* próprio da Oceania e Ásia (Gräser, De Hoog, *et al.*, 2000; Da Silva Lacaz *et al.*, 2002).

Os dermatófitos já foram descritos desde o século XIX. O gênero *Microsporum* foi descrito pelo húngaro David Gruby em 1843 (Gruby, 1843). O gênero *Tricophyton* foi descrito

pelo sueco *Malmsten*, em 1845, e *Epidermophyton* foi individualizado por Sabouraud em 1907. De início, só foram descritas as formas assexuadas ou imperfeitas de tais microrganismos. Com numerosas espécies, algumas delas foram mal identificadas e colocadas com nomes duvidosos (Da Silva Lacaz *et al.*, 2002).

Logo veio um enorme avanço no estudo dos dermatófitos com a prática do isolamento de algumas espécies do solo pelo método da isca (hairbait), utilizado pela primeira vez pelo micólogo belga Raymond Vanbreuseghem em 1952 (Vanbreuseghem, 1952). Esse processo permitiu descobrir o estágio perfeito ou teleomorfo dos gêneros *Trichophytom* e *Microsporum*, com as denominações iniciais de *Nannizzia* e *Arthroderma*. Mais tarde, concluíram que *Arthroderma* e *Nannizia* são congenêricos, considerando *Nannizzia* sinônimo de *Arthroderma* (Rippon, 1988; Da Silva Lacaz *et al.*, 2002). Porém, de Hoog et al. sugeriram voltar a desagregar e a considerar Nannizzia como estágio perfeito das espécies do complexo *A. gypseum* (De Hoog *et al.*, 2017).

As espécies das formas anamorfas dos dermatófitos são diferenciadas pela macromorfologia das colônias e a presença ou ausência de macro e microconídios (número, forma, tamanho, dimensões, etc). As formas teleomorfas são diferenciadas pelos seus gimnotécios. Todas as espécies de *Arthroderma* conhecidas são heterotálicas, com exceção da espécie homotálica *A. ciferri* (Da Silva Lacaz *et al.*, 2002; Metin e Heitman, 2017).

Como já foi descrito, as diferentes espécies de dermatófitos podem se agrupar com base na sua preferência ecológica. Estes agrupamentos ecológicos são observados nos clusters filogenéticos obtidos por mtDNA ou rDNA (Gräser, De Hoog, *et al.*, 2000; Gräser *et al.*, 2008; Kanbe, 2008; Mochizuki *et al.*, 2017; Verrier e Monod, 2017). Estes estudos sugerem que inicialmente todos eles eram moradores do solo e costumavam prosperar na queratina espalhada na terra. O contato direto com animais e o homem fez com que eles se adaptassem ao seu estilo de vida zoofílico ou antropofílico respectivamente. Uma teoria sugere que o *Homo sapiens* moderno desenvolveu a capacidade de apresentar menos pêlos corporais para evitar os parasitas, podendo estar entre eles os dermatófitos. Para se adaptar a esta carência de pêlos em humanos, os dermatófitos que evoluíram desde a terra e os animais até o homem, perderam gradualmente sua capacidade de produzir macroconídios para se transmitir e reproduzir, produzindo artroconídios, que possibilitaram uma transmissão por contato direto. Os macroconídios podem se pegar aos pêlos dos animais para se dispersarem, podendo ser este animal um portador sem sintomas da

doença (ex: gatos); estes macroconídios são raros ou ausentes nas cepas antropofílicas. Devido ao número crescente de pessoas no planeta, o fungo evoluiu perdendo a capacidade de produzir esporos adaptados a se transmitir por animais peludos para produzir esporos mais resistentes frente às novas condições (arthrosporos ou chlamidosporos). O gene ou genes responsáveis por disparar esta inibição ou inativação da produção de macroconídios ainda estão sendo pesquisados para estas espécies antropofílicas (Da Silva Lacaz *et al.*, 2002; Sharma e Gräser, 2011; Cafarchia *et al.*, 2013).

Aspectos morfológicos.

Trichophyton spp. tem micro e macroconídios de paredes lisas. Os macroconídios crescem geralmente lateralmente, diretamente da hifa ou pequenos pedículos, e podem ser de paredes delgadas ou grossas, clavadas ou fusiformes, porém, podem ser poucas ou estar ausentes. Os microconídios são esféricos, piriformes ou clavados, mas podem ter formas irregulares. A presença de microconídios distingue este gênero de *Epidermophyton*, e os conídios de paredes lisa, em sua maioria sésseis (sem órgão de fixação) separa-o de *Microsporum* (Chen *et al.*, 2011).

*Microsporum a*presenta macroconídios holotálicas produzidas por cada espécie que podem ser propágulos bi ou multiseptados que contribuem na transmissão do fungo. O número de células em um único macroconídio representa o mesmo número de sub – propágulos, tendo todos eles a capacidade de germinar para formar um talo individual. *Microsporum spp*. geralmente produz abundantes macroconídios em ágar Malta ou ágar Sabouraud suplementados com pêlo humano ou de cavalo, com exceção daquelas espécies que não costumam produzir macroconídios (Sharma e Gräser, 2011).

As colônias de *E. flocossum* são geralmente de lento crescimento, com cor que pode variar de amarelo mostarda até algumas tonalidades de verde. A superfície inicialmente é camurçada no princípio é aplanada, mas logo fica com elevações e dobras no centro. O reverso tem uma cor amarela bronzeada. *E. flocossum* com o passar do tempo fica rapidamente branca, macia e estéril (Liu e Cole, 2011). Microscopicamente observam-se hifas septadas e hialinas, macroconídios e células semelhantes a clamidósporos, não se observando nunca a presença de microconídios. *E. flocossum* diferencia-se de *T. ajelloi* por sua inabilidade de perfurar o cabelo e por sua tolerância tardia ao NaCl numa concentração de 7 %. Além disso, os macroconídios de *E. flocossum* são mais curtos que os de *T. ajelloi*. De maneira geral, *E. flocossum* também se

diferencia de outras espécies de *Microsporum* ou *Tricophyton* pela ausência de microconídios (Da Silva Lacaz *et al.*, 2002; Liu e Cole, 2011).

Existe certa similaridade na morfologia dos macrosporos dos dermatófitos, sendo multiseptados e apresentando um mecanismo rhexolitico para a liberação dos esporos. A similaridade em sua morfologia é provavelmente o resultado de uma adaptação evolutiva para a vida dermatofítica, teoria suportada pela proximidade filogenética e morfológica ao fungo degradador de queratina *Chrysosporium spp*., fungo que apresenta aleuroconídios indistinguíveis ao microscópio dos gêneros *Microsporum* e *Tricophyton,* sugerindo que *Chrysosporium* é um precursor ancestral destes fungos dermatófitos (Sharma e Gräser, 2011). Aliás, *Chrysosporium* já foi considerado como um quarto gênero dos dermatófitos (Gräser *et al.*, 2008).

Ecologia e epidemiologia.

Os *Trichophyton* patogênicos podem ser antropofílicos ou zoofílicos (afetam primariamente animais, como gatos, cavalos, vacas, ovelhas e camelos). Geralmente as espécies geofílicas não são patogênicas, salvo algumas exceções (Chen *et al.*, 2011; Da Costa *et al.*, 2013; Sun *et al.*, 2014; Surendran *et al.*, 2014; Nweze e Eke, 2016; Mozes *et al.*, 2017; Zhan e Liu, 2017).

Dentre os dermatófitos, *Microsporum* está bem adaptado ao solo devido a sua ampla produção de macroconídios (com exceção de algumas espécies que evoluíram recentemente como antropofílicas), existindo também várias espécies zoofílicas de importância médica como *M. canis*.(Sharma e Gräser, 2011). O gênero *Epidermophyton* só é constituído pela espécie antropofílica *E. flocossum*. A segunda espécie do gênero chamada *E. stockdaleae*, agora é considerada como uma variante de *T. ajelloi* (Liu e Cole, 2011).

Distribuição de espécies.

A distribuição de espécies do gênero *Trichophyton* varia segundo a geografia. As tendências globais tendem a uma substituição progressiva de espécies zoofílicas, por exemplo, *T. verrucosum*, por espécies antropofílicas, em especial *T. rubrum*. O aumento de *T. rubrum* como a causa mais comum de tinea pedis, tinea unguium (onicomicose), tinea cruris e tinea corporis é devido à urbanização, práticas socioculturais e a imigração. A persistência das espécies zoofílicas está associada com infecções epidémicas em animais da rua ou domésticos. *T. rubrum* é a espécie predominante na Europa Central e do Norte, Estados Unidos e outros países desenvolvidos, onde a prevalência de tinea pedis e onicomicose é elevada. Por outro lado, *T. verrucosum* e *T.*

violaceum são as espécies mais frequentes no Meio Oriente e África do Norte, sendo endêmico da África Central o fungo *T. soudanense*. A presença de algumas espécies também varia com o local de infecção. De 50 a 90% das infecções por tinea capitis no Reino Unido, Estados Unidos, Brasil e Jamaica são causadas por *T. tonsurans*, já na China ocidental, *T. violaceum* é responsável por 41,9% das infecções. Tinea capitis provocada por *T. schoenleinii* é endémica na África do Sul e Oriente Médio, enquanto é provocada por *T. concentricum* no Sudeste da Ásia e Polinésia (Chen *et al.*, 2011).

A África é considerada como o berço da civilização, onde se encontra a origem do *Homo sapiens* (Quintana-Murci *et al.*, 1999). Os dermatófitos ancestrais, incluindo os jovens antropofílicos, podem ter se originados na África. Se isto for verdadeiro, a separação de duas linhagens para transformarem-se em duas espécies diferentes do complexo *M. canis* ocorreu depois das migrações dos primeiros humanos da África até a Ásia, permitindo a especiação alopátrica, e resultando nas espécies *M. audonii* e *M. ferrugineum* (Gräser *et al.*, 2006). A distribuição cosmopolita de algumas espécies de dermatófitos (não associado com aves) tem sido facilitada pelo humano e os animais que ele transporta (domésticos ou selvagens) (Sharma e Gräser, 2011).

Manifestações clínicas.

As descrições das manifestações clínicas das infecções por *Trichophyton* e outros dermatófitos são abundantes na bibliografia (Kawai *et al.*, 2014; Lourdes *et al.*, 2014; Sun *et al.*, 2014; Surendran *et al.*, 2014; John *et al.*, 2016; Hay, 2017; Trocoli Drakensjo *et al.*, 2017). As formas clínicas da doença no humano dependem da espécie (por exemplo, as espécies zoofílicas causam uma reação inflamatória mais aguda que as espécies antropofílicas), o local do corpo afetado e do estágio imune do hospedeiro (Da Silva Lacaz *et al.*, 2002). Infecções restringidas à pele ou outros tecidos superficiais são típicas, mas podem ocorrer infecções mais complexas (Lourdes *et al.*, 2014). As infecções dos dermatófitos são classificadas de acordo com a região do corpo afetada e o grau de queratinização: Tinea corporis e tinea cruris (tinea do corpo e inguinal), Tinea pedis (tinea do pé), Tinea capiti (tinea do cabelo), Tinea unguium (onicomicose). Também existem formas invasivas da doença (granuloma de Majocchi, invasões locais com envolvimento subcutâneo, e disseminação nos ossos, nódulos linfáticos, fígado e sistema nervoso central) (Da Silva Lacaz *et al.*, 2002; Chen *et al.*, 2011; Lourdes *et al.*, 2014; Trocoli Drakensjo *et al.*, 2017)

E. flocossum é uma espécie antropofílica, não afetando os animais.No caso de *Trichophyton* e *Microsporum* isto não acontece, causando, estes gêneros, um considerável número de doenças em animais selvagens e domésticos (Albano *et al.*, 2013; Da Costa *et al.*, 2013).

Diagnóstico de laboratório convencional.

Tradicionalmente o diagnóstico é feito pela associação das características microscópicas com isolamento em meio de cultura. A identificação baseada no cultivo é um método lento (até 4 semanas) e, frequentemente impreciso devido ao pleomorfismo fenotípico do fungo e pouco treino do pessoal. Porém, estes métodos ainda são muito aplicados devido aos baixos custos. Também os métodos moleculares estão sendo empregados cada vez mais, possibilitando uma rápida identificação da espécie e detectando DNA próprio do fungo em amostras clínicas diretamente (Da Silva Lacaz *et al.*, 2002; Kondori *et al.*, 2013; Mochizuki *et al.*, 2017; Verrier e Monod, 2017).

Métodos diagnósticos baseados na Biologia Molecular.

Com as técnicas moleculares disponíveis, o diagnóstico da infecção causada por dermatófitos a partir de uma amostra clínica ou outros materiais, se faz de maneira rápida e exata. Estudos comparativos mostram as vantagens destas técnicas em relação ao diagnóstico convencional (Paugam *et al.*, 2013). Num estudo, foram analisadas 191 amostras de cabelo e pele detectando-se por PCR 37% dos isolados em um menor tempo em comparação aos métodos convencionais e com uma especificidade de 84 e 86% para pele e pêlo respectivamente (Kondori *et al.*, 2013). As características morfológicas, tanto micro como macroscópicas são úteis porque podem focar as técnicas moleculares a aplicar sugerindo o uso de primers específicos para cada espécie (Gräser *et al.*, 2006).

Têm sido desenhadas técnicas moleculares para identificar certeiramente isolados de meios de cultura, para detectar diretamente DNA de dermatófitos em amostras clínicas e para estudar as variações das cepas em estudos epidemiológicos. Os fatores que podem influenciar na detecção de DNA em amostras clínicas incluem um correto processamento da espécime, um protocolo de extração de DNA e o desenho de uma técnica de PCR (seleção do alvo e primer adequados) (Chen *et al.*, 2011).

A seleção de uma sequência alvo é um passo crítico para a sensibilidade e o nível de identificação (gênero ou espécie) do ensaio. Os ensaios que marcam genes de várias cópias

(multi-copy) são geralmente mais sensíveis que aqueles focados em genes de cópia única (single-copy). O alvo mais amplamente usado é o complexo de genes do rDNA. Embora a região variável do locus da SSUr DNA permita uma delineação filogenética, são a região altamente variável ITS1/2 e o seu locus intermediário 5.8S rDNA, as sequências especialmente úteis para a identificação de espécies fúngicas, incluindo dermatófitos. O PCR geralmente se combina com outros métodos (RFLP: restriction fragment length polimorphism) naqueles ensaios onde se usa uma cópia única (ex: CHSI, TOP2 e protease alcalina 1) para obter uma adequada especificidade e sensibilidade (Gräser, Kuijpers, *et al.*, 2000; Yoshida *et al.*, 2006; Chen *et al.*, 2011; Cafarchia *et al.*, 2013; Verrier e Monod, 2017).

Métodos para a detecção e/ou identificação de dermatófitos.

Dentro das técnicas criadas para a identificação de culturas de dermatófitos encontram-se a análise PCR-RFLP, amplificação por PCR de um loci genético específico, seguido de análise de sequências, PCR em tempo real e outros métodos baseados em sondas. Técnicas similares têm sido aplicadas à detecção de DNA destes fungos em amostras clínicas, embora muitos ensaios de PCR sejam só moderadamente sensíveis ou não podem fornecer informação relativa à identificação de espécies (Chen *et al.*, 2011; Cafarchia *et al.*, 2013).

Atualmente, as técnicas mais comumente usadas para reconhecer espécies de *Microsporum* devido a sua simplicidade e baixos custos são ITS-PCR-RFLP. O sequenciamento ITS-PCR é capaz de diferençar espécies muito relacionadas, mas é uma técnica não muito fácil de realizar e também mais dispendiosa. As duas técnicas envolvem a amplificação do DNA alvo num termociclador para incrementar sua visibilidade num gel de eletroforese. Na RFLP, o DNA amplificado usualmente de um sítio selecionado do genoma (ex: região ITS do rDNA) pode ser cortado com enzimas de restrição para revelar um padrão específico da espécie similar ao das cepas de referência, embora às vezes não aconteça assim. As desvantagens do RFLP é que espécies relacionadas como *M. canis* e *M. ferrugineum* não são diferençadas e uma grande quantidade de produto de PCR é necessário. Uma melhor alternativa é o sequenciamento da região ITS e a posterior comparação com as sequências de referência encontradas nas bases de dados nucleotídicas (NCBI). Também se recomenda o uso da base de dados validada do CBS (http://www.cbs.knaw.nl) (Sharma e Gräser, 2011; Verrier e Monod, 2017).

Estudos moleculares aplicados à filogenia.

A aplicação de marcadores filogenéticos moleculares tem questionado o reconhecimento dos 4 gêneros anamorfos morfologicamente diferençados de *Arthroderma*. Especificamente, o conceito de espécie filogenética tem proporcionado dados, onde o gênero *Trichophyton* encontra-se separado dos gêneros *Epidermophyton* e *Microsporum* com algumas espécies de *Chrysosporium* intercaladas com alguns *Trichophyton spp*. geofílicos. A próxima relação genética entre estes gêneros anamorfos indica que a distinção de espécies baseadas nas características morfológicas pode não ser tão certa. Está claro que algumas espécies previamente consideradas únicas baseadas nas diferenças morfológicas são espécies diferentes no nível molecular ou vice-versa. Exemplo disto são as espécies *T. rubrum* e o fungo endémico da África *T. soudanense*, que têm espaçadores transcritos internos (ITS, do inglês: internal trascribed spacer) idênticos dentro do DNA ribosomal (rDNA). Situação similar ocorre com algumas espécies do complexo *T. mentagrophytes* reagrupadas, atendendo aos marcadores moleculares como *T. interdigitale* (Gräser *et al.*, 2006; Gräser *et al.*, 2008). Portanto, algumas espécies estão sendo reagrupadas com base nestes estudos (Gräser, El Fari, *et al.*, 1999; Gräser, Kuijpers, *et al.*, 1999; Gräser, Kuijpers, *et al.*, 2000; Simpanya, 2000; Gräser *et al.*, 2008; Chen *et al.*, 2011; De Hoog *et al.*, 2017).

O polimorfismo dentro de numerosos loci em vários genes tem sido utilizado para definir as relações filogenéticas entre espécies de dermatófitos, incluindo: DNA mitocondrial (mDNA), gene da actina, gene da quitina sintase 1 (CHS1), gene da topoisomerase II (TOP2), gene da manganeso superóxido dismutase e subunidades menores (SSU), ITS, subunidades maiores (LSU) e espaçadores intergênicos (IGS) dos genes do rDNA (Gräser, De Hoog, *et al.*, 2000; Chen *et al.*, 2011; Liu e Cole, 2011; Sharma e Gräser, 2011; Cafarchia *et al.*, 2013):

A análise destes loci tem dado como resultado um refinamento nos avanços da taxonomia. Muitas espécies classificadas como uma única espécie baseadas em sua morfologia agora são "separadas" como distintas espécies e outras que estavam separadas juntam-se para constituir uma única espécie. Um alto grau de clonalidade observa-se em algumas espécies, por exemplo, espécies dentro do complexo *T. rubrum* /*T. violaceum*, levando a tratar estas entidades clonais como espécies separadas (Gräser, De Hoog, *et al.*, 2000; Chen *et al.*, 2011).

Em um estudo polifásico, Gräser *et al*. (2008), combinando -conceitos morfológicos e biológicos ("mating", ecológicos, e interação com o hospedeiro) e filogenéticos de espécies,

recomendaram uma nova taxonomia para dermatófitos antropofílicos e zoofílicos, baseada em quatro complexos de espécies principais. Três deles têm associação com o estágio teleomorfo *Arthroderma* (complexo *A.vanbreuseghemii*, *A. gypseum*, *A. otae*), no entanto, o complexo *T. rubrum* é exclusivamente assexual (Gräser *et al.*, 2008). De Hoog et al. (2017) propôs um outro rearranjo dentro da taxonomia desagrupando novamente a espécie *T. interdigitale*, mudando espécies dentro do complexo *T. rubrum* / *T. violaceum* e introduzindo novamente o estágio sexual *Nanizzia* vinculado às espécies previamente classificadas dentro do complexo *A. gypseum* (De Hoog *et al.*, 2017).

Notavelmente, a classificação taxonômica varia em relação aos estudos moleculares aplicados. Portanto, determinar as barreiras de espécies dentro dos dermatófitos pode requerer futuras avaliações usando pesquisas moleculares alternativas como são as sequências codificadoras de inteins PRP8 (Theodoro *et al.*, 2008; Theodoro e Bagagli, 2009; Theodoro *et al.*, 2011).

Inteins

Um intein é uma inserção dentro da proteína hospedeira que é removida usando um processo de autosplicing durante a pós-tradução. Este autosplicing protéico deve incluir um corte preciso da proteína interna (Intein) e a junção das regiões próximas do intein da proteína externa (Extein) formando uma ligação peptídica (Gogarten *et al.*, 2002; Butler *et al.*, 2006; Perler, 2008). Por questões de simplicidade, chamaremos intein também à sequência de DNA que codifica para o intein.

Nas bactérias e archaea os inteins estão distribuídos em proteínas envolvidas na replicação, recombinação, reparo ou transcrição do DNA (DNA / RNA polimerase, helicase e topoisomerase). Nos eucariotas, estão localizados principalmente em ATPase da membrana vacuolar (Intein VMA), RNA de transferência (Intein ThrRS) e a proteína PRP8 (Intein PRP8). Muitos inteins são encontrados principalmente em domínios altamente conservados de proteínas essenciais ("house-keeping"). A remoção da sequência intein de um domínio conservado é um processo delicado e altamente eficiente, uma vez que uma deleção imprecisa levaria à inativação das proteínas. Como resultado, inteins são mantidos nesses domínios (Novikova *et al.*, 2016).

Além de apresentarem motivos de splicing para realizar o processo de autosplicing, muitos inteins também podem ter uma homing endonuclease (HE). Estas HE podem ser classificadas em diferentes famílias, dependendo dos sítios ativos ou motivos constitutivos

distintivos (ex: grupo LAGLIDADG e His-Cys box), sendo a família LAGLIDADG a família mais comum presente em inteins PRP8. HE similares podem ser encontradas também nos introns do Grupo I (Burt e Koufopanou, 2004; Chevalier *et al.*, 2008).

A HE pode causar uma conversão gênica, permitindo que uma célula heterozigota para o intein seja transformada em uma célula homozigota. Para iniciar a conversão gênica, a HE codificada pelo intein reconhece um local específico numa grande sequência de DNA alvo que corresponde a um local alélico desocupado. Em seguida, a HE causa uma ruptura cromossômica de cadeia dupla na sequência alvo desocupada e a maquinaria de reparação do DNA celular do hospedeiro repara a ruptura utilizando o alelo que contém o intein como modelo. O alelo recentemente ocupado não é mais um alvo da HE porque o local alvo foi separado pela inserção. Portanto, os inteins são elementos móveis que ocupam locais específicos no genoma. Quando são copiados para um alelo desocupado, eles ainda são retidos no alelo doador (Burt e Koufopanou, 2004).

Propôs-se, com base em informações de estudos do intein VMA, que ao nível da população, o processo de replicação "super-mendeliano" causado pela HE poderia aumentar a frequência do intein no pool genético de uma espécie sexuada até ser fixado no genoma (Butler *et al.*, 2006). Quando o intein atinge uma fixação no genoma, então não haverá alelos desocupados. Os movimentos intra-específicos do intein não ocorrerão e as forças de seleção não funcionarão na função HE. Como as forças de seleção não estão mais atuando sobre a HE, então ela se tornará inativa por acumulação de mutações aleatórias. De acordo com a previsão anterior, muitos inteins de VMA mostraram possuir uma HE não funcional (Butler *et al.*, 2006). Estudos de substituições sinônimas e não –sinônimas mediante o uso da bioinformática mostraram ser úteis para determinar essa atividade (Gonzales *et al.*, 2002; Butler *et al.*, 2006; Theodoro *et al.*, 2011).

Foram sugeridos que os inteins passam por ciclos recorrentes de: i) Transferência horizontal para novos genomas, ii) Fixação na população receptiva por processo de homing, iii) Degeneração do gene *HE* pela ausência de sequências alvo e iv) Perda eventual do intein por processo de deleção. Este ciclo se conhece como ciclo homing (Burt e Koufopanou, 2004). Aproximadamente 15% dos inteins conhecidos carecem de domínios HE (mini-inteins). Estes mini-inteíns provavelmente foram originados de deleções de inteins inteiros e têm um comprimento de 130-200 aa com blocos de sequências conservadas em cada extremo do intein.

Como os inteins completos têm uma região central com uma sequência HE, então eles são maiores do que os mini-inteins com um comprimento de 360-550 aa (full-length intein) (Perler, 2008).

Por estudos de mutagênese e estruturas de cristais 3D foi descrito que as funções Homing e Splicing estão completamente separadas. Portanto, o domínio HE é um componente silencioso do intein que mostra pouca ou nenhuma relação estrutural ou funcional com o domínio splicing (Chong e Xu, 1997; Duan *et al.*, 1997). Os mini-inteins podem ser considerados como um exemplo extremo de perda ou corrupção da HE. A perda da função homing não permitirá a replicação do intein intra ou interespecífica por homing, restringindo a evolução das mini-inteins e a sua distribuição filogenética (Burt e Koufopanou, 2004; Butler e Poulter, 2005; Butler *et al.*, 2006).

Os inteins podem ser encontradas em vários organismos procarióticos e em alguns unicelulares eucarióticos. Por um tempo, o único intein descrito em eucariota foi o intein VMA, encontrado no gene da ATPase vacuolar de alguns hemiascomicetos como *Saccharomyces cerevisiae* (Hirata et al., 1990; Kane et al., 1990). Estes inteins estão exatamente localizados no mesmo local (VMA-a) dentro do gene hospedeiro *VMA1* e são referidas como inteins alélicos. Todos os inteins VMA têm uma HE LAGLIDADG, o que sugere a possibilidade de uma transmissão horizontal frequente entre espécies de leveduras de ascomicetos, embora o mecanismo para essa transmissão permaneça desconhecido (Butler *et al.*, 2006).

Os fatos da presença de um segundo intein em eucariotas foram publicados nos primeiros anos do atual milênio. O intein alélico PRP8 de *Cryptococcus neoformans* e *Cryptococcus gatii* carece de HE. O mini-intein de *C. neoformans* é traduzido dentro da proteína PRP8 que é codificada pelo gene nuclear *prp8* (Butler *et al*., 2001; Butler e Poulter, 2005). A proteína PRP8 é uma proteína chave do spliceosoma que é altamente conservada, e atua no processo de splicing do mRNA (Alberts, 2008). A função splicing do intein deve permanecer funcional embora a função da HE seja corrompida ou perdida a fim de remover o intein do precursor da proteína PRP8. Se o intein não é removido é provável que exista um spliceosoma não funcional e o fungo não será capaz de processar o mRNA (Butler *et al.*, 2006).

O intein PRP8 foi descrito em: *Paracoccidioides brasiliensis* (onygenales), *Botritys cinerea* (helotiales), seção clavati e fungos fumigati (Eurotiales) e muitas outras espécies dentre estas três ordens. Muitos dos inteins PRP8 são mini-inteins, ao contrário dos inteins VMA de

leveduras que sempre têm uma HE. Nas três ordens de fungos euascomicetos, o intein prp8 foi comprovado que pode apresentar ou não uma HE. Ainda nestes fungos, existem espécies que não contêm intein, mesmo quando são muito próximas filogeneticamente de outras que apresentam intein. (Butler *et al.*, 2006). Por essa razão, a distribuição filogenética do intein PRP8 é muito enigmática e futuros estudos podem ainda ser feitos para dar resposta a esta distribuição aparentemente aleatória. Novikova et al. com o suporte da bioinformática confirmaram uma tendência à distribuição de inteins entre categorias funcionais de proteínas com forte preferência por uma única rede de funções contendo proteínas do processo de replicação em bactérias e archaeas (Novikova *et al.*, 2016).

Muitas proteínas envolvidas no processo de replicação não ortólogas que são funcionalmente equivalentes em bactérias e archaea carregam inteins, sugerindo uma retenção seletiva de inteins em proteínas de funções especificas. Os Inteins se agrupam não apenas em proteínas com papéis relacionados, mas também em unidades funcionais específicas dessas proteínas, como o domínio ATPase (Novikova *et al.*, 2016). Esta distribuição peculiar não se enquadra plenamente no modelo que descreve inteins como elementos parasitas (Burt e Koufopanou, 2004).

No modelo parasítico, a dinâmica evolutiva do intein é vista principalmente através da mobilidade do intein mediante a HE como o principal fator de aquisição ou perda do intein. Embora a HE seja essencial para invasão e disseminação do intein em uma população, a dinâmica da HE não explica a distribuição observada de inteins entre proteínas com categorias funcionais específicas. Propõe-se que o domínio splicing do intein poderia atuar como um sensor ambiental que se adapta a um nicho específico podendo aumentar a chance do intein se tornar fixo em uma população. A retenção do intein pode ser benéfica sob certos estresses ambientais atuando como botões de pânico, inibindo de forma reversível redes específicas. Isto poderia explicar a distribuição do intein em proteínas específicas (Novikova *et al.*, 2016).

O intein PRP8, primeiramente descrito em fungos basidiomicetos (*Cryptococcus neoformans* e *C. gatii*), está amplamente representado dentro dos membros da ordem Onygenales, sendo muito comum na família Ajellomycetaceae (*P. brasiliensis*, *Paracoccidioides lutzii*, *Histoplasma capsulatum*). Alguns artigos caracterizam o intein PRP8 e estabelecem as relações filogenéticas com o uso desta região nuclear. Foi comprovado que as diferenças entre as

sequências do intein refletem a relação filogenética entre as espécies hospedeiras dos gêneros (Butler *et al.*, 2001; Theodoro *et al.*, 2008; Theodoro *et al.*, 2011; Theodoro *et al.*, 2013).

Justificativa

Considerando que as infecções causadas por dermatófitos são indistinguíveis umas das outras e são facilmente confundidas com dermatites e outras infecções superficiais fúngicas não dermatofíticas, é imperativo identificar o agente causal até o nível de espécie para um tratamento efetivo. Além disso, uma identificação ao nível de cepa é útil para estudos epidemiológicos (Gräser *et al.*, 2006). Solução que poderia se alcançar usando os métodos da biologia molecular (Verrier e Monod, 2017).

O intein PRP8 tem sido meramente mencionado dentro da família Arthrodermataceae (*M. gypseum*. *T. rubrum*, *T. interdigitale*) (Novikova *et al.*, 2016), sendo só caracterizado em *M. canis (Perler, 2000)*. Porém, nenhuma caracterização foi feita dentro dos dermatófitos dos gêneros *Trycophyton* e *Epidermophyton* e nunca foi avaliado na literatura este intein como marcador filogenético para dermatófitos.

Objetivo Geral:

- Caracterizar o Intein PRP8 em fungos dermatófitos.

Objetivos específicos

- Identificar as cepas molecularmente utilizando regiões nucleares ribossomais e as sequências do próprio intein.
- Determinar as relações de filogenia usando a região nuclear do intein PRP8 e a sua sequência aminoacídica.
- Identificar regiões polimórficas dentro do intein que permita a identificação dos dermatófitos mediante ensaio de PCR/eletroforese.

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2. Molecular identification and phylogenetical analysis of dermatophyte fungi from Latin America.

Summary:

Dermatophytes constitute a complex group of fungi, comprised of by the genera Trichophyton, Epidermophyton and Microsporum. They have the ability to degrade keratin and cause human and animal infections. Molecular techniques have made their identification faster and more accurate, and allowed important advances in phylogenetic studies. We aim to identify molecularly and to determine the phylogenetic relationships in dermatophyte fungi from Brazil and other Latin American countries, using DNA sequencing of the nuclear ribosome regions ITS1-5.8S-ITS2 and D1/D2. DNA of 45 dermatophytes was extracted and amplified by PCR for identification at the species level by sequencing of those ribosomal regions. The software MEGA 6.0 was used to establish the phylogenetic relationships via the Maximum Likelihood method. Out of 45 strains, 43 were identified by ITS (95.5%) and 100% by D1/D2 sequencing. Two strains could not be identified by ITS. Phylogenetic analyses separated the genera Trichophyton and *Microsporum*, which presented an uncertain relationship with *Epidermophyton floccosum*, depending on the ribosomal marker. Both regions can provide efficient identification of dermatophytes, whereas phylogenetic analysis revealed complex relations among dermatophyte fungi.

Key words: Dermatophytes, molecular identification, phylogeny, Arthrodermataceae.

Introduction.

Dermatophytes are keratinophilic fungi belonging to the family Arthrodermataceae (Onygenales, Ascomycota), and include dozens of related species, differentiable mainly by their anamorph or asexual forms into three classical genera – *Trichophyton*, *Microsporum* and *Epidermophyton* – though recent molecular phylogenetic studies indicate the existence of a fourth genus, denominated *Chrysosporium*[1]. Most of these organisms have the ability to invade keratinised tissues (skin, hair and nails) resulting in a condition called dermatophytosis or tinea.

While *Trichophyton* and *Microsporum* genera contain distinct anthropophilic, zoophilic and geophilic species, according to their ability to cause infection primarily in humans or other animals, or to occur preferably as free-living residents in soil, respectively, the genus *Epidermophyton* encompasses only one species, *E. floccosum*, which affects only humans[2–4].

These ecological groups present congruence with phylogenetic clusters obtained by mitochondrial DNA or the DNA coding for ribosomal RNA (mtDNA or rDNA).[1,2,5,6] These studies corroborate the previous idea that the ancestors of these species inhabited the soil and were able to spread due to their ability to induce keratin degradation. Thus, their subsequent direct contact with animals and man led them to adapt to their zoophilic or anthropophilic lifestyle[1,7].

Although infections caused by dermatophytes are relatively difficult to distinguish from each other and are easily mistaken for other non-dermatophytic superficial fungal infections, it is imperative to identify the causal agent at the species level, both for more effective treatment and for epidemiological purposes[1]. Traditionally, the diagnosis has been made by association of parasitic microscopic aspects with the isolates in culture. Despite being a low-cost approach, the culturing procedure is slow (up to 4 weeks) and often inaccurate due to the fungal phenotypic polymorphism and lack of trained staff. That is why molecular methods are promising for accurate species identification and direct fungal DNA detection in clinical and environmental samples[3,8–12].

The anamorph species forms of dermatophytes are relatively well differentiated by the macromorphology of the colonies and by the presence or absence of macro- and microconidia (number, shape and size), whereas the teleomorph forms are differentiated by their gymnothecium. These morphological and biological features have enabled the establishment of the different species under morphological and biological species concepts[1,8,10,12]. The application of molecular phylogenetic markers has cast doubt on the recognition of the four morphologically differentiated anamorphic genera within the teleomorph Arthroderma genus. When the phylogenic species concept is applied, some *Chrysosporium* species cluster with geophilic *Trichophyton* species. However, these dermatophyte-related *Chrysosporiums* species are molecularly distant from the *C. merdarium*-type species. Therefore, additional studies and taxonomical debates are necessary to establish future changes in the taxonomy of these microorganisms[1]. In addition, the phylogenetic species concept has provided data indicating that

the genus *Trichophyton* is paraphyletic, since it is split by *Microsporum* and *Epidermophyton* genera. The close genetic relationship between these anamorph genera hampers the distinction of dermatophyte species, based only on morphological characteristics. It is clear that some species previously considered distinct by morphological techniques are not so different at the molecular level and vice versa[1,8,10,12,13].

Several molecular markers have been applied in phylogenetic studies to establish the limits of dermatophyte species[13–18]. The nuclear non-coding ribosome regions ITS (internal transcribed spacer) 1 and ITS 2 are highly variable and most often used for fungal identification[13,18–20]. In fact, ITS sequencing has been considered the gold standard for identifying dermatophytes[1]. Other nuclear ribosomal regions, such as D1/D2 located in the large ribosomal subunit 28S, are also useful[21–24]. Herein, we aimed to identify molecularly and to determine the phylogenetic relationships in dermatophyte fungi from Brazil and other Latin American countries, using DNA sequencing of the nuclear ribosome regions ITS1-5.8S-ITS2 and D1/D2.

Materials and methods.

Strains.

Forty-five strains from Brazilian collections were used: 23 from the Institute for Tropical Medicine of the University of São Paulo (IMT-SP), 19 from the Institute Lauro Souza Lima (ILSL), Bauru, São Paulo and three provided by Prof. Laerte Ferreiro from the Federal University of Rio Grande do Sul (UFRGS). The origin and designation of each strain are shown in Table 1.

Molecular analyses.

Fungal DNA was extracted via the phenol-chloroform method as described by Gräser et al. [25]. Molecular identification was performed by PCR reactions using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG – 3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC – 3′) for the

ITS1-5.8S-ITS2 region and NLS1 (5′- GCATATCAATAAGCGGAGGAAAAG – 3′) and NLS4 (5′- GGTCCGTGTTTCAAGACGG – 3′) for the D1/D2 regions[26]. PCR was performed, using a Veriti Thermocycler (Applied Biosystems, Foster City, USA) with Phusion_ (Thermo Scientific, Foster City, USA) PCR kit requirements (25 µl of reaction mixture - containing 3 µl of genomic DNA, 0.2 mM dNTP, 5 µl 5X Phusion HF Buffer, 0.5 µM of each primer, 3% DMSO, 0.02 $U\mu l^{-1}$

Phusion DNA Polymerase and water to complete the reaction volume). The thermal cycling conditions were: 98 °C for 1.5 min followed by 35 cycles at 98 °C for 0.5 min, 55 °C (ITS region) and 60 °C (D1/D2 region) for 0.5 min, 72 °C for 1 min and a final extension at 72 °C for 10 min.

Table1: Species identification by DNA sequencing of the ITS1-5.8S-ITS2 and D1/D2 regions. The final identification was established according to Gräser et al, 2008 [1].

Strain	Received as: (by morphological criteria)	Source / Site of infection	ITS1-5.8-ITS2		D1/D2		Final Identification
			Ident. %	Strain / Accession Number	Ident.%	Strain / Accession Number	
28	*T. mentagrophytes*	Human / *Tinea* unguium	100	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
B1	*T. mentagrophytes*	Human / *Tinea* unguium	100	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
B3	*T. mentagrophytes*	Human / Tinea corporis	100	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
B4	*T. mentagrophytes*	Human / Tinea corporis	100	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
B5	*T. mentagrophytes*	Human / Tinea corporis	100	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
B6	*T. mentagrophytes*	Human / Tinea corporis	100	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
B10	*T. mentagrophytes*	Human / Tinea unguium	100	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
B13	*T. mentagrophytes*	Human / Tinea corporis	100	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
B16	*T. mentagrophytes*	ATCC 9533 (Human) / N.I	100	*T. interdigitale* ATCC 9533 / KC146353.2	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*

433	*T. mentagrophytes*	Human / Tinea corporis	100	*T. interdigitale* ATCC 28146 / KJ722759.1	100	*T. interdigitale* ATCC 8757 / KJ606152.1	*A. vanbreuseghemii*
B19	*T. mentagrophytes var. granulosum.*	ATCC 62905 / N.I	99	*T interdigitale* ATCC 62904 / KJ606096.1	100	*T interdigitale* ATCC 62904 / KJ606144.1	*T. interdigitale* (zoophilic)
B2	*T. rubrum*	Human / Tinea unguium	100	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
B11	*T. rubrum*	Human / Tinea corporis	99	*T. interdigitale* ATCC 24952 / KJ606110.1	100	*T. interdigitale* ATCC 28146 / KJ722761.1	*T. interdigitale*
31	*T. rubrum*	Human / Tinea corporis	100	*T. rubrum* ATCC 28191 / KF 278457.1	100	*T. rubrum* ATCC 28191 / KF 278472.1	*T. rubrum*
B7	*T. rubrum*	Human / Tinea corporis	99	*T. rubrum* ATCC 28191 / KF 278457.1	100	*T. rubrum* ATCC 28191 / KF 278472.1	*T. rubrum*
B8	*T. rubrum*	Human / Tinea unguium	100	T. rubrum ATCC 28191 / KF 278457.1	100	T. rubrum ATCC 28191 / KF 278472.1	*T. rubrum*
B9	*T. rubrum*	Human / Tinea unguium	100	*T. rubrum* ATCC 28191 / KF 278457.1	100	*T. rubrum* ATCC 28191 / KF 278472.1	*T. rubrum*
B12	*T. rubrum*	Human / Tinea corporis	100	*T. rubrum* ATCC 28191 / KF 278457.1	100	*T. rubrum* ATCC 28191/KF 278472.1	*T. rubrum*
B17	*T. rubrum*	ATCC 28189 (Human) / Tinea corporis	100	*T. rubrum* ATCC 28188/ EU200370.1	100	*T. rubrum* ATCC 28191 / KF 278472.1	*T. rubrum*
B20	*T. rubrum*	Human / N.I	100	*T. rubrum* ATCC 28188 / EU200370.1	100	*T. rubrum* ATCC 28191 / KF 278472.1	*T. rubrum*

368	*T. violaceum*	Human / N.I	100	*T. rubrum* ATCC 28191 / KF 278457.1	100	*T. rubrum* ATCC 28191 / KF 278472.1	*T. rubrum*
1013	*T. violaceum*	N.I /N.I	N.A	Not amplified.	99	*T.rubrum* ATCC MYA-4607 / KJ606169.1	*T. rubrum*
1024	*T. soudanense*	N.I / N.I	N.A	Not amplified.	100	*T. gourvilii* CBS 170.65 / EF078484.1 (synonymous of *T. rubrum*, African population)	*T. rubrum* (African population)
84	*E. floccosum*	Human / Tinea corporis	100	*E. floccosum* ATCC 52066 / EF631604.1	100	*E. floccosum* ATCC 26072 / AY213697.1	*E. floccosum*
198	*E. floccosum*	Human / Tinea corporis	100	*E. floccosum* ATCC 52066 / EF631604.1	100	*E. floccosum* ATCC 26072 / AY213697.1	*E. floccosum*
431	*M. canis*	Human / Tinea capitis	100	*M.canis* ATCC MYA-4605 / GU291265.1	100	*M.canis* ATCC 23828 / Y213708.1.	*M.canis*
747	*M.canis*	Human / N.I	100	*M.canis* ATCC MYA-4605/ GU291265.1.	100	*M.canis* ATCC 23828 / Y213708.1.	*M.canis*
830	*M. canis*	Human / Tinea capitis	100	*M.canis* ATCC MYA-4605/ GU291265.1.	100	*M.canis* ATCC 23828 / Y213708.1.	*M.canis*
B14	*M. canis*	Human / Tinea capitis	100	*M.canis* ATCC MYA-4605/ GU291265.1.	99	*M.canis* ATCC 23828 / Y213708.1.	*M.canis*
RS4	*M. canis*	Dog / Skin lesion	100	*M.canis* ATCC MYA-4605/ GU291265.1.	100	*M.canis* ATCC 23828 / Y213708.1.	*M.canis*
RS5	*M. canis*	Cat / Skin lesion	99	*M.canis* ATCC MYA-4605/ GU291265.1.	100	*M.canis* ATCC 23828 / Y213708.1.	*M.canis*

841	*M. equinum*	Human / N.I	100	*M.canis* ATCC MYA-4605/ GU291265.1.	100	*M.canis* ATCC 23828 / Y213708.1.	*M.canis*
483	*M. audouinii*	Human / N.I	99	*M.audouinii* IHEM 16242 / FJ360692.1	100	*M. audoinii* ATCC 10216 / EF078482.1	*M. audoinii*
541	*M. gypseum*	Human / N.I	99	*A. incurvatum* MCCF 1687 / KC784360.1	100	*A. incurvatum* CBS 174.64 / AY176738.1	*M. gypseum*/ *A. incurvatum*
586	*M.gypseum*	Human / Tinea corporis	99	*A. incurvatum* MCCF 1687 / KC784360.1	100	*A. incurvatum* CBS 174.64 / AY176738.1	*M. gypseum* / *A. incurvatum*
589	*M. gypseum*	Human / N.I	99	*A. incurvatum* MCCF 1687 / KC784360.1	100	*A. incurvatum* CBS 174.64 / AY176738.1	*M. gypseum* / *A. incurvatum*
B15	*M. gypseum*	Human / Tinea corporis	99	*A. incurvatum* MCCF 1687 / KC784360.1	100	*A. incurvatum* CBS 174.64 / AY176738.1	*M. gypseum* / *A. incurvatum*
435	*M. gypseum*	Human / Tinea corporis	100	*M. gypseum* ATCC MYA-4604/GU291264.1	100	*A. gypseum* ATCC 22925 / AY176727.1	*M. gypseum* / *A. gypseum*
546	*M. gypseum*	Soil	100	*M. gypseum* ATCC MYA-4604/GU291264.1	100	*A. gypseum* ATCC 22925 / AY176727.1	*M. gypseum* / *A. gypseum*
613	*M.gypseum*	Human / Tinea capitis	99	*M. gypseum* ATCC MYA-4604/GU291264.1	100	*A. gypseum* ATCC 22925 / AY176727.1	*M. gypseum* / *A. gypseum*
1019	*M.persicolor*	N.I / N.I	100	*M. persicolor* CBS 139.323 / KP636536.1	100	*M. persicolor* CBS 139.323 / KP636537.1	*M. persicolor*
1025	*M. fulvum*	N.I / N.I	100	*T. longifusum* ATCC 22397 / EF631616.1 (synonymous of *M. fulvum*)	100	*A. fulvum* CBS 287.55 / EF078483.1	*M. fulvum*
119	*T. tonsurans*	Human / N.I	100	*T. tonsurans* ATCC MYA-4608/KJ606101.1	100	*T. tonsurans* ATCC MYA-4608/ KJ606149.1	*T. tonsurans*
429	*T. tonsurans*	Human / Tinea	100	*T. tonsurans*	100	*T. tonsurans*	*T. tonsurans*

		corporis		ATCC MYA-4608/ KJ606101.1		ATCC MYA-4608 / J606149.1	
RS3	*T. ajelloi*	Cow / Skin lesion	100	*A. uncinatum* ATCC 28454 / EF631614.1	99	*A. uncinatum* gen for 28SrRNA/ AB075329.1	*T. ajelloi*

N.I: Not informed; N.A: Not amplified.

The PCR products were detected as a single band of 500–700 bp for ITS1-5.8s-ITS2 and 600 bp for D1/D2 by 1.5% agarose gel electrophoresis. The amplicons were purified by using EXOSAP-IT® (Affymetrix, Cleveland, OH, USA) and submitted to sequencing of both strands in the 3500 analyzer (Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. Sequencing edition, and phylogenetic analysis were accomplished using the software MEGA v6.0.27 Molecular identification was performed by BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) from the database of the National Center for Biotechnology Information (NCBI). Each species was identified from the best-scoring reference sequence of the blast output with an identity >98% compared to the query sequence. Additionally, each sequence of the ITS1-5.8S-ITS2 region was blasted using the ITS sequences supplied by Gräser et al. 2008 [1] as the subject sequences. Both the ITS1-5.8s-ITS2 and D1/D2 regions were used for species identification based on the criteria of Gräser et al. 2008 [1] for nomenclature of dermatophyte species.

Three phylogenetic analyses were performed with aligned ITS1-5.8S-ITS2, D1/D2 and joining D1/D2 and ITS1-5.8S-ITS2 (D1/D2+ ITS) sequences of the isolates, also were included American Type Culture Collection (ATCC), Centraal Bureau voor Schimmelcultures (CBS) and other sequences deposited in the genbank database (accession numbers in Table 1). Alignment was accomplished through Clustal W. The Maximum Likelihood (ML) method[28] was performed by applying Tamura 3-parameters[29] for ITS1-5.8SITS2 and D1/D2+ ITS sequences with bootstrap of 500 replicates with random additions[30]. *Chrysosporium merdarium* was used as the outgroup because it is a distant species with high divergence from other *Chrysosporium* species related to geophilic dermatophytes [1] (accession number AJ390384 for ITS1-5.8SITS2 sequences and KF225604 for D1/D2 sequences). The same method but applying Kimura 2-parameters[31] was applied for D1/D2 partial sequences.

The ITS1-5.8S-ITS2 and D1/D2 nucleotide sequences reported in this paper were submitted to GenBank_ database under accession numbers: KT354595–KT354634 (ITS) and KT443097–KT443143 (ITS and D1/D2).

Results

All strains amplified the D1/D2 region; however, two strains (1024 and 1013) did not amplify the region ITS1-5.8S-ITS2. Final identification of each strain is displayed in Table 1. The additional species identification by blasting the ITS sequences supplied by Gräser et al. [1] for each species (data not shown in Table 1) was entirely concordant with the identification of both nuclear ribosomal regions, with DNA identities of 97% for *Microsporum gypseum*/*Arthroderma incurvatum* and 99 or 100% for all other remaining strains. This last check-out using the sequences suggested by Gräser was particularly useful to determine the exact classification of the strain 433 as *Arthroderma vanbreuseghemii* with ITS identity of 100% and to confirm the zoophilic or anthropophilic type of *T. interdigitale*.

A high number of *T. interdigitale* (eight strains) were morphologically misnamed as *T. mentagrophytes* (Table 1). All previous samples of morphologically identified *T. rubrum* (nine strains) and *T. violaceum* (two strains) were molecularly confirmed as *T. rubrum*, while one *T. soudanense* strain (#1024) was correctly identified by its D1/D2 region as *T. gourvili*, which, according to Graser et al. [1] might be classified as *T. rubrum* in the African Population.

Of the seven strains morphologically identified as *M. gypseum* (shown in Table 1), four were identified as *A. incurvatum* (strains 541, 586, 589 and B15) and three as *A. gypseum* (strains 435, 546 and 613). On the other hand, strains of two species, namely *M. fulvum* and *M. persicolor*, which also might be clustered in the *M. gypseum* complex, according to Gräser et al. [1] presented complete concordance between morphological and molecular identification.

Only four strains were misdiagnosed following morphological criteria, involving species of the *T. rubrum*/ *T. violaceum* and *A. vanbreuseghemii* complexes (Table 1). Two strains morphologically identified as *T. rubrum* (strain B2 and B11) were unambiguously identified by nuclear ribosomal markers as *T. interdigitale*, whereas two strains previously identified as *T. violaceum* (strain 368 and 1013) were molecularly identified as *T. rubrum*. Strain 1013 did not amplify the ITS region; the molecular identification for this strain was confirmed only by the D1/D2 region.

A phylogenetic tree using the ML method for both regions ITS1-5.8S-ITS2 and D1/D2 is represented in Fig. 1 and Fig. 2 respectively. The acceptable bootstraps values confirm the robustness of each tree. A main difference can be noted in relation to the position of the genus *Epidermophyton*. *E. floccosum* clusters with the *T. rubrum/T. violaceum* complex by D1/D2

region analysis (Fig. 2) and with the *A. gypseum* complex by ITS1-5.8S-ITS analysis (Fig. 1). Joining the ITS and D1/D2 regions, in a concatenated analysis, we have observed that *E. floccosum* remains associated with the *M. gypseum* complex but less closely related to and no longer clustering with *M. persicolor* (Fig. 3). Within the studied dermatophytes, *T. ajelloi* was confirmed as the most ancient species and shown to be related to *A. otae* complex, confirmed by all three phylogenetic constructions.

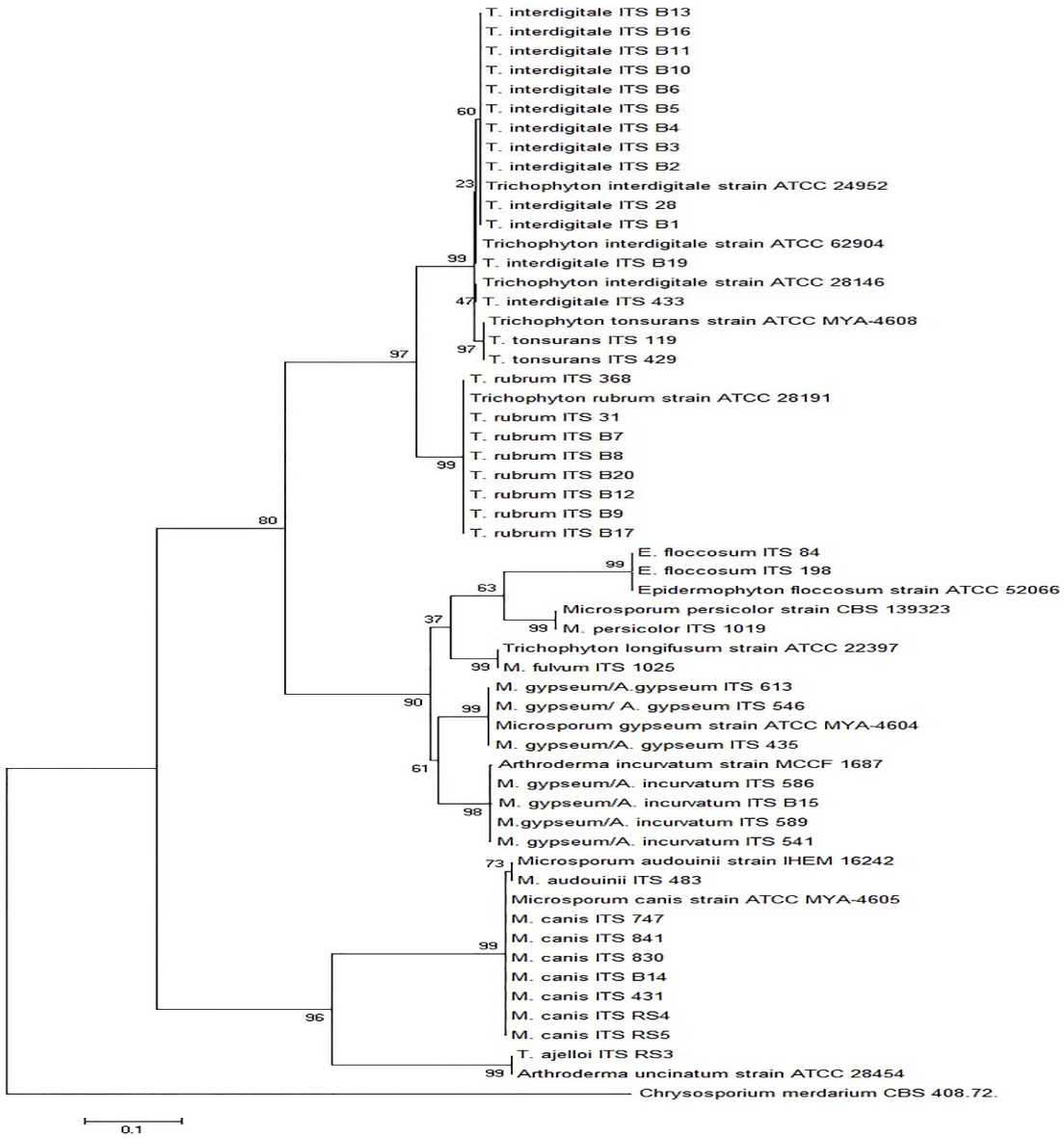

Figure 1: The evolutionary history using ITS1-5.8S-ITS2 region sequences was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500

replicates) is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA v6.0.

Figure 2: The evolutionary history using D1/D2 region sequences was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA v 6.0

Figure 3: The evolutionary history using concatenated D1D2 and ITS1-5.8S-ITS2 region sequences was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA v 6.0.

Discussion

Sequencing of the ITS region is considered the gold standard molecular method for identifying dermatophytes since it relies on the most complete database that can be used for dermatophytic identification[1]. The ITS region was shown to be an excellent marker by virtue of its ability to distinguish even between closely related dermatophytic species[8,10,12,13,32]. On the other hand, the D1/D2 region is traditionally used to identify yeast[23,24]; however, Ninet et al. [33] proposed an accurate identification of dermatophytes, using the D1/D2 region. Studies focusing on this region have been conducted in dermatophytes and other filamentous fungi[13,34], but have been less often applied when compared to the ITS region. The results of identification using region D1/D2 as a molecular marker proved to be efficient and congruent with the ITS identification. Despite being an apt marker for identification of dermatophytic species, some species such as *A. uncinatum*, *M. persicolor* and *M. fulvum* have a reduced number of deposited sequences for this region. To identify a fungus in this database, it is necessary to pay attention to the names of the different species (anamorph/teleomorph) because the submitted sequences represent different points of view and historical periods.

The former taxonomy of the teleomorphic *A. Vanbreuseghemii* complex has been controversial, and includes the anamorphic species *T. tonsurans*, *T. equinum* and *T. interdigitale*. *T. interdigitale* possesses some anthropophilic or zoophilic strains, which were classified as part of the *T. mentagrophytes* complex (Ex: *T. mentagrophytes* var. *interdigitale*, *T. mentagrophytes* var. *nodulare*, *T. mentagrophytes* var. *mentagrophytes*, *T. mentagrophytes* var. *granulosum*). Nowadays, thanks to molecular applications, all these variants were grouped within a single species (*T. interdigitale*), being the only dermatophyte with a marked ecological bifurcation that can be efficiently differentiated by applying these techniques[1,7,13,35,36]. Some changes also have been proposed for species of the *T. rubrum*/*T. violaceum* complex (*T. soudanense*, *T. gourvilii*, *T. megninii* and *T. raubitschekii*) that might be molecularly grouped into the single species *T. rubrum*. This complex has two anthropophilic species *T. rubrum* (with African and European population) and *T. violaceum*[1,37,38]. Therefore, it is highly advisable to be updated on changes in complex taxonomic groups such as dermatophytes to correctly name the species according to the current taxonomical definitions. The species of both complexes are extremely pleomorphic and have presented a broad range of clinical manifestations[3,8,39,40], leading to misdiagnosis of the causative agent by morphological criteria.

Regarding *M. gypseum* complex, it was corroborated the existence of the two teleomorphic species (*A. gypseum* and *A. incurvatum*) occurring among *M. gypseum* anamorphic strains, requiring molecular techniques to separate these two sexual species[1,13]. This fact confirms that the morphological species concept in *M. gypseum* does not have biological support.

The use of molecular markers for species identification can be correlated with the biological species concept in geophilic species, since none of the geophilic teleomorphic species shows ITS sequence identities higher than 97%[1,7]. Analysing our geophilic species, 83% ITS sequence identity can be observed between *M. gypseum*/*A. gypseum* and *T. ajelloi* (strains 435 and RS3). Even for closer geophilic species within the same complex, such as *M. gypseum*/*A. gypseum* and *M. gypseum*/ *A. incurvatum* (strains 435 and 541), the ITS sequence identity herein observed was 96%, but never higher than 97%. This finding was not observed in animal-associated dermatophytes, most notably in those species that no longer mate[1,7]. The ITS identity observed between *T. tonsurans* and *T. interdigitale* (strains 119 and B1) was 98%, vs. 99% between closer species such as *M. canis* and *M. audoinii* (strains 747 and 483). This is one of the limitations of the biological species concept. Considering that some dermatophyte species do not perform sexual reproduction, then the application of the biological species concept could be problematic for biosystematic analysis, thus rendering the phylogenetic species concept more adequate for that purpose.

Although dermatophytes as a group have a monophyletic origin[6], the phylogenetic analyses performed herein have confirmed that all dermatophytic genera are paraphyletic[1,8,32]. This characteristic is reflected in all three phylogenetic trees in which the *Trichophyton* genus is split by the genera *Microsporum* and *Epidermophyton*, suggesting that ecology is a determining factor in the evolution of dermatophytes[1,7,8,12,13]. The current taxonomy of dermatophytes is strongly based on morphologic characters, which might have suffered similar selective pressures to induce convergent evolution. The application of molecular approach has shown that morphological grouping does not reflect the true evolutional history of dermatophytes fungi.

Although there are no phylogenetic constructions on dermatophytes using D1/D2 region sequences at the consulted bibliography, the constructed phylogenetic tree (Fig. 2) shows that this region is also useful for phylogenetic approaches since it reflected a similar evolutionary history when compared with the ITS1-5.8S-ITS2 region constructions (Fig. 1), at least in relation to the analysed species.

Cafarchia et al. [32] by studying dermatophytes fungi among rabbits and farm workers, found that *E. floccosum* is closer to the *M. gypseum* complex, both for chitin synthase 1 and the ITS region. On the other hand, Graser et al. [1] report that *E. floccosum* is closer to the *T. rubrum*/*T. violaceum* and *A. vanbreuseghemii* complexes, according to the ITS region. The analysis of mitochondrial genomes of dermatophytic species revealed that the organization of the genes encoding the 25 tRNA is highly conserved in all mitochondrial genomes, and that *E. floccosum* and *T.rubrum* present the largest cluster of tRNA between rnl and cox1, interrupted and divided into two sub-clusters by a 2.5 kb fragment of DNA[6]. It can be concluded that the genus *Epidermophyton* remains in an uncertain position between the genera *Microsporum* and *Trychophyton*, a scenario that demands additional phylogenetic studies.

Fossil evidence establishes the emergence of Ascomycota fungi approximately 400 million years ago (mya)[41]. Based on this calibration, it was estimated that the lineage of dermatophytes might have diverged about 32–50 mya[6]. Remarkably, it appears that *T. ajelloi* diverged from a common ancestor much earlier than other dermatophytic species corresponding to the geophilic characteristics of *T. ajelloi*[6,34]. Studies based on sequences of the D1/D2 region show that the species *T. ajelloi* and *T. terrestre* are phylogenetically separated from the rest of the dermatophytes with early divergences from a common ancestor[34]. The closeness between *T. ajelloi* and *M. canis* can be observed in mitochondrial genomes, in which these two fungi are the only species without any overlap of a base and no interruption between genes of the nad4L-nad5-nad2 gene unit[6].

The taxonomy of dermatophytes is varying due to not only new molecular studies but also to the application of new approaches[13]. The D1/D2 region has been demonstrated a suitable region for phylogenetic constructions on dermatophytes. Therefore, determining the barriers of species in dermatophytes may require further assessment through additional studies on new molecular markers.

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3. PRP8 intein in dermatophytes: Evolution and species identification.

Abstract.

Dermatophytes are keratinophilic fungi belonging to the family *Arthrodermataceae*. Despite having a monophyletic origin, its systematics has always been complex and controversial. Sequencing of nuclear ribosomal ITS and D1/D2 rDNA has been proposed as an efficient tool for identifying species in this group of fungi while multilocus analyses have been used for Phylogenetic Species Recognition. However, the search for new markers, with sequence and size variation, which enable species identification in only one PCR step, is very attractive. Inteins seems to fulfill these characteristics. They are self-splicing genetic elements present within housekeeping coding genes, such as *PRP8,* that codify the most important protein of the spliceosome. The PRP8 intein has been described for *Microsporum canis* in databases but has not been studied in dermatophytes in any other published work. Thus, our aim was to determine the potential of this intervening element for establishing phylogenetic relationships among dermatophytes and for identifying species. It was found that all studied species have a full-length PRP8 intein with a Homing Endonuclease belonging to the family LAGLIDADG. Phylogenetic analyses were consistent with other previous phylogenies, confirming *Epidermophyton floccosum* in the same clade of the *Arthroderma gypseum* complex, *Microsporum audouinii* close to *M. canis*, differentiating *A. gypseum* from *Arthroderma incurvatum*, and in addition, better defining the *Trichophyton interdigitale* and *Trichophyton rubrum* species grouping. Length polymorphism in the HE region enables identification of the most relevant *Microsporum* species by a simple PCR-electrophoresis assay. Intein PRP8 within dermatophytes is a powerful additional tool for identifying and systematizing dermatophytes.

Keywords: Dermatophytes, PRP8 intein, Phylogeny, Identification.

Introduction.

Dermatophytes are filamentous fungi present in soil, humans and other animals. This broad capacity for living in different habitats enabled their classification according to their way of life as geophilic, zoophilic and anthropophilic [1-3]. Three genera are classically established: *Trichophyton*, *Microsporum* and *Epidermophyton*, based on morphological characteristics. They belong to the order Onygenales and possess keratinases for degrading keratinized tissues in animals and humans, causing superficial and cutaneous lesions on skin, hair or nails [4-8].

The taxonomy of this fungal group has been controversial and research studies have been published, some of which unify different species into only one and others that propose new cryptic species complexes [9-12]. Besides morphological, physiological and reproductive studies to classify dermatophytes [13-17], molecular biology has elucidated some systematic issues by applying the phylogenetic species concept [3, 11, 12, 18, 19].

Some variable genomic markers have been proven useful to identify and classify dermatophytes, such as ITS (internal transcribed spacer) 1 and ITS 2 from the nuclear non-coding ribosomal RNA and D1/D2 regions located in rRNA from the large ribosomal subunit 28S [3, 11, 19, 20]. Other genomic variable regions, such as inteins, that have been applied for identifying fungal species [21-24], have never been explored in dermatophytes.

Inteins are intervening protein splicing elements firstly identified in 1990 in the *VMA* (ATPase Vacuolar Membrane) gene in *Saccharomyces cerevisiae* [25, 26]. Since then, additional studies have applied much effort into understanding the mechanism for intein splicing from the precursor protein, mainly concerning the amino acids (aa) residues, from both inteins and exteins (intein flanking sequences), indispensable for this function. [27-30]. Therefore, protein-splicing is defined as the excision of the intein coupled with ligation of N and C-terminal exteins, which leads to an active and functional protein [31-33]. If this mechanism is not

performed in a highly efficient manner, it could result in a miss-functional or totally inactive protein (extein). A deficient splicing mechanism may lead to intein loss by selective pressures [22, 34, 35].

Inteins are considered ancient elements and can reach new targets by means of a homing endonuclease (HE), generally found within the intein sequences, splitting the splicing domain into N- and C-terminals domains. Inteins are often located in conserved host protein motifs probably by the conservation of the HE specific DNA recognition sites, which possess sufficiently length to promote a DSB (double strand break) in one specific location of the genome, commonly an empty and cognate allele [36-39]. Inteins that contain a HE are denominated full-length inteins and are larger than mini-inteins (those containing only the splicing domain) [22]. Several conserved blocks have been characterized for both homing and splicing domains, these blocks represent some residues responsible for a catalytic or structural function. Blocks A, B, F and G are located in the intein splicing domain, whilst blocks C, D, E and H are in the HE [22, 34]. It was determined that protein- splicing and endonuclease domains have separate active sites. Mutations of the intein active-site residues do not inhibit endonuclease function and vice versa [40, 41].

Among fungal inteins, the PRP8 intein is the most widespread, despite its sporadic distribution [22]. The *PRP8* gene encodes for the Splicing Factor Prp8, a core protein of the spliceosome complex responsible for removing introns from mRNA [42]. Many fungal species containing the PRP8 intein are important human pathogens. The PRP8 intein of *C. neoformans* and *C. gattii,* for instance, lacks HE, being a mini-intein, while other species, such as *P. brasiliensis*, *A. fumigatus* and *H. capsulatum* possess a full-length PRP8 intein, with a HE belonging to LAGLIDADG family [21-24]. The LAGLIDADG protein family was the first intron-encoded proteins to be identified and biochemically characterized. It shows

conservation of a ten-residue sequence motif and is the most diverse of the homing endonuclease families [39].

It has been observed that the differences between the intein sequences reflect the phylogenetic relationship between the host species, being useful for establishing proper systematic classification of complex species groups [23, 24, 43-46]. The only fully described intein sequence in dermatophytes is the PRP8 intein of *M. canis* [47]; thus, no other intein sequences have been characterized for this group of fungi. Therefore, we aim to characterize the molecular aspects of PRP8 inteins in representative species of dermatophytes in order to better define their phylogenetic relationship and also to evaluate whether this intein might be used as a simple molecular marker, based on PCR-electrophoresis, for distinguishing species.

Materials and methods

Strains:

Forty strains of 11 different dermatophyte species were used for PRP8 intein sequencing. Strains were previously obtained, described and identified by DNA sequencing of the nuclear ribosome regions ITS1-5.8S-ITS2 and D1/D2 by Garcia Garces et al, (2016)[19]. Although some current nomenclature changes have been adopted for dermatophytes [12], the species denomination herein employed was based on Gräser et al. (2008)[3]. Both denominations might be observed at table 1 for each strain.

Table 1: Strain identification by using nuclear ribosomal regions (ITS1-5.8S-ITS2 and D1/D2) and PRP8 intein DNA sequences.

Strain	Molecular identification (ITS1-5.8S-ITS2 and D1/D2 region)*	Identification by PRP8 intein (Blasting at Whole-genome shotgun contigs database, WGS)			Final identification by Gräser et al, 2008[3]. (By de Hoog et al. 2017[12])
		Base pairs (bp)	% Identity	Identification	
747	*M. canis*/ *A. otae*	1686	100	*A. otae* strain CBS 113480	*M. canis*/ *A. otae.* (*M. canis*)
841	*M. canis*/ *A. otae*	1686	100	*A. otae* strain CBS 113480	*M. canis*/ *A. otae.* (*M. canis*)
431	*M. canis*/ *A. otae*	1686	100	*A. otae* strain CBS 113480	*M. canis*/ *A. otae.* (*M. canis*)
830	*M. canis*/ *A. otae*	1686	100	*A. otae* strain CBS 113480	*M. canis*/ *A. otae.* (*M. canis*)
483	*M. audouinii*	1686	98	*A. otae strain CBS 113480*	*M. audouinii* (*M. audouinii*)
RS3	*T. ajelloi*/ *A. uncinatum*	1455	85	*A. otae strain CBS 113480*	*T. ajelloi* / *A. uncinatum* (*A. unicinatum*)
589	*M. gypseum*/ *A. incurvatum*	1449	87	*M. gypseum* CBS 118893	*M. gypseum*/ *A. incurvatum* (*Nannizzia incurvata*)
586	*M. gypseum*/ *A. incurvatum*	1449	87	*M. gypseum* CBS 118893	*M. gypseum*/ *A. incurvatum (Nannizzia incurvata)*
541	*M. gypseum*/ *A. incurvatum*	1449	87	*M. gypseum* CBS 118893	*M. gypseum*/ *A. incurvatum (Nannizzia incurvata)*
B15	*M. gypseum*/ *A. incurvatum*	1449	87	*M. gypseum* CBS 118893	*M. gypseum*/ *A. incurvatum* (*Nannizzia incurvata*)
613	*M. gypseum*/ *A. gypseum*	1473	100	*M. gypseum* CBS 118893	*M. gypseum*/ *A. gypseum* (*Nannizzia gypsea*)
435	*M. gypseum*/ *A. gypseum*	1473	100	*M. gypseum* CBS 118893	*M. gypseum*/ *A. gypseum* (*Nannizzia gypsea*)
546	*M. gypseum*/ *A. gypseum*	1473	100	*M. gypseum* CBS 118893	*M. gypseum*/ *A. gypseum* (*Nannizzia gypsea*)
1019	*M. persicolor*/ *A. persicolor*	1473	84	*M. gypseum* CBS 118893	*M. persicolor*/ *A. persicolor* (*Nannizzia persicolor*)
1025	*M.fulvum*/ *A. fulvum*	1500	89	*M. gypseum* CBS 118893	*M.fulvum*/ *A. fulvum* (*Nannizzia fulva*)
198	*E. floccosum*	1503	85	*M. gypseum* CBS 118893	*E. floccosum* (*E. floccosum*)

84	*E. floccosum*	1503	85	*M. gypseum* CBS 118893	*E. floccosum* (*E. floccosum*)
433	*A. vanbreuseghemii*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. mentagrophytes*)
B19	*T. interdigitale (zoophilic)*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. mentagrophytes*)
28	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B1	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B13	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B3	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B4	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B5	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B6	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B16	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B10	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
B11	*T. interdigitale*	1503	100	*T. interdigitale* H6	*T. interdigitale* (*T. interdigitale*)
368	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum* (*T. rubrum*)
31	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum* (*T. rubrum*)
1013	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum* (*T. rubrum*)
B7	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum* (*T. rubrum*)
B8	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum* (*T. rubrum*)
B9	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum* (*T. rubrum*)

B12	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum* (*T. rubrum*)
B17	*T. rubrum*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum* (*T. rubrum*)
1024	*T. rubrum (African population)*	1503	100	*T. rubrum CBS 202.88*	*T. rubrum* (*T. soudanense*)
119	*T. tonsurans*	1503	99	*T. tonsurans* CBS 112818	*T. tonsurans* (*T. tonsurans*)
429	*T. tonsurans*	1503	99	*T. tonsurans* CBS 112818	*T. tonsurans* (*T. tonsurans*)

*Identification made by Garcia Garces et al. 2016 [19].

PRP8 intein sequencing:

DNA samples previously obtained by Garcia Garces et al. (2016) were used for intein sequencing[19]. Extein regions flanking the intein were determined by aligning dermatophyte *PRP8* DNA sequences from the GenBank database of the National Center for Biotechnology Information (NCBI) and Centraal Bureau voor Schimmelcultures (CBS) dermatophyte database for primer design. The primers EXTDF 5′ ATC AAG YTG CAC CTG GAA AC 3′ and EXTDR 5′ GAC AAG TCA RAG ACA TGA TGG 3′ (IDT, Coralville, IO, USA) were designed to anneal at the extein *PRP8* sequences flanking the PRP8 intein. PCR was performed in a Veriti Thermocycler (Applied Biosystems, Foster City, CA, USA) using GoTaq® Green Master Mix (Promega, Madison, WI, USA), PCR kit requirement (25 µl of reaction mixture-containing 3 µl of genomic DNA at 600ng/µl, 12.5 µl of GoTaq® Green Master Mix 2X, 1.4 µl of each primer at 10µM and nuclease-free water to complete the reaction volume). Thermal cycling conditions were: 98 °C for 2 min followed by 35 cycles at 98 °C for 0.5 min, 60 °C for 0.5 min, 72 °C for 1 min and a final extension at 72 °C for 10 min.

PCR products were detected as a single band of 1400–1700 bp by 1.5% agarose gel electrophoresis. The PCR mixture was purified by using EXOSAP-IT (Affymetrix, Cleveland, OH, USA) and submitted to sequencing of both strands in the 3500 analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Analysis of the PRP8 intein sequences.

After sequencing, sense and antisense sequences (approximately 600 to 700bp) were aligned and a second set of internal primers was designed in order to complete the whole intein sequence. Primers INTDF 5′TCC TKG GRC TYT GGC TTG G 3′ and INTDR 5′CGG ACR GYR CGC GAA TTG 3′ (IDT, Coralville, IO, USA) were used for amplifying the internal regions of the PRP8 intein. PCR cycling conditions, detection by electrophoresis,

purification and sequencing were the same but using 3.0 µl of previous PCR reaction, instead of genomic DNA, for amplification and 58 °C as the annealing temperature.

The entire PRP8 intein DNA sequence was constructed by aligning the four previously obtained sequences (2 from primers EXTD and 2 from primers INTD) for each strain. Sequences edition, alignment with Clustal W, conversion to protein sequence and further phylogenetic analysis were executed by using the software MEGA v 6.0 [48].

Additionally all PRP8 DNA sequences were submitted for molecular identification by Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nlm.nih.gov/BLAST/) from the NCBI site to confirm the previous ITS1-5.8s-ITS2 and D1/D2 identification [19] and to validate the sequencing edition. Each species was identified from the best-scoring reference sequence of the blast output with an identity >98% compared to the query sequence (only for previously deposited species). The Whole-Genome Shotgun contigs database (WGS) was useful for that purpose. BLASTp was also used in order to confirm identification by protein sequences.

Comparative studies between obtained sequences and other PRP8 intein sequences [22] allowed us to locate both the splicing and homing-endonuclease conserved blocks. The PRP8 intein nucleotide sequences reported in this paper were submitted to the GenBank database under accession numbers: KX463279-81, KX497134-59 and KX510280-90.

The protein sequences for each species were exported to a CLC sequencer viewer v7.7.1 available at www.clcbio.com (QIAGEN Aarhus A/S, Aarhus, Denmark); alignment was carried out and exported to PDF (Supplementary Material 1).

The dS/dN rates were calculated for both splicing and HE conserved block domains by using Synonymous–non-synonymous mutation rates between sequences containing ambiguous nucleotides (Syn-SCAN) [49] available at

https://hivdb.stanford.edu/pages/synscan.html, in order to infer the conservation and possible function of both domains.

Experimental identification of putative introns in the PRP8 inteins from *M. canis* and *M. audouinii*.

Strains 747 and 830 of *M. canis* and *483* of *M. audouinii* were used for RNA extraction by using TRIZOL® reagent (Invitrogen, Cincinnati, USA) and treated with DNase I (Thermo Scientific, Carlsbad, CA, USA). The mRNA was converted to cDNA by using the IScript™cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), according to the manufacturer's instructions. Primers McaP241F 5´CGC TGT CTT CTT GAA CAA TTC G 3´ and McaP242R 5´AGC TGA TGT CCA TCA ACT CGG 3´ (IDT, Coralville, IO, USA) were designed from intein regions surrounding the supposed intron. Three µl of cDNA samples was amplified by PCR, visualized in gel electrophoresis, purified and sequenced following the same requirements for the intein sequencing but with an annealing temperature of 55 °C.

Phylogenetic analysis.

Two phylogenetic analyses were performed. Alignment was accomplished through Clustal W alignment method for DNA sequences[50] and a Point Accepted Mutation (PAM) method for protein alignment[51]. The Maximum Likelihood (ML) method [52] was performed by applying the Kimura Parameter [53] for intein DNA sequences with bootstrap of 1000 replicates with random additions [54]. The same method, but with Jones-Taylor-Thornton Parameter [55], was applied for protein sequences using the same bootstrap values. For both constructions, *Chrysosporium queenslandicum* (accession number: LJPI00000000.1) and *Uncinocarpus reesii* (accession number: CH476615.1) were used as out-groups because applying phylogenetic analysis of the PRP8 gene (data not shown) it can be seen that *Uncinocarpus reesii* is the closest species out of dermatophyte containing a PRP8 intein. *Chrysosporium*

queenslandicum is the only *Chrysosporium* species available in NCBI databases with a whole genome deposited (intein never previously described), whereas a close relationship has been proven between *Chrysosporium* species and dermatophytes [3].

The data and phylogenetic constructions were deposited at treeBASE (www.Treebase.org). Access: http://purl.org/phylo/treebase/phylows/study/TB2:S20861 and http://purl.org/phylo/treebase/phylows/study/TB2:S20862 for analyses related to DNA sequences and protein sequences, respectively.

Identifying *Microsporum* species by PRP8 intein.

Two PCR electrophoreses were designed for differentiating clinical important *Microsporum* species. Figure 1 summarizes the proposed diagram for this experiment. The first assay was performed by using the primers HE1F 5´TTC CTK GGR CTY TGG CTT GG 3´ and HE2R 5´ADY AAA CCD GCR AGG ACG G3´ (IDT, Coralville, IO, USA) to distinguish *M. canis* and M. *audouinii* from the rest of the other clinically important dermatophytes, including the genus *Trichophyton*. PCR was performed following the same conditions employed for amplifications of inteins, with an annealing temperature of 58 °C. The electrophoresis assay was carried out in a 2.5% agarose gel for 2.5 h at 120 V.

The second assay was designed for differentiating within the *A. gypseum* species complex herein studied. A third reverse primer HE3R 5´GTC CAG ATC RYC CWC TTT SG 3´ (IDT, Coralville, IO, USA) was designed, using as forward the same primer used for *M. canis*/ *M. audouinii* identification (HE1F). The annealing temperature was 57 °C and the bands were visualized by electrophoresis in a 2.5% agarose gel after 2h at 120V.

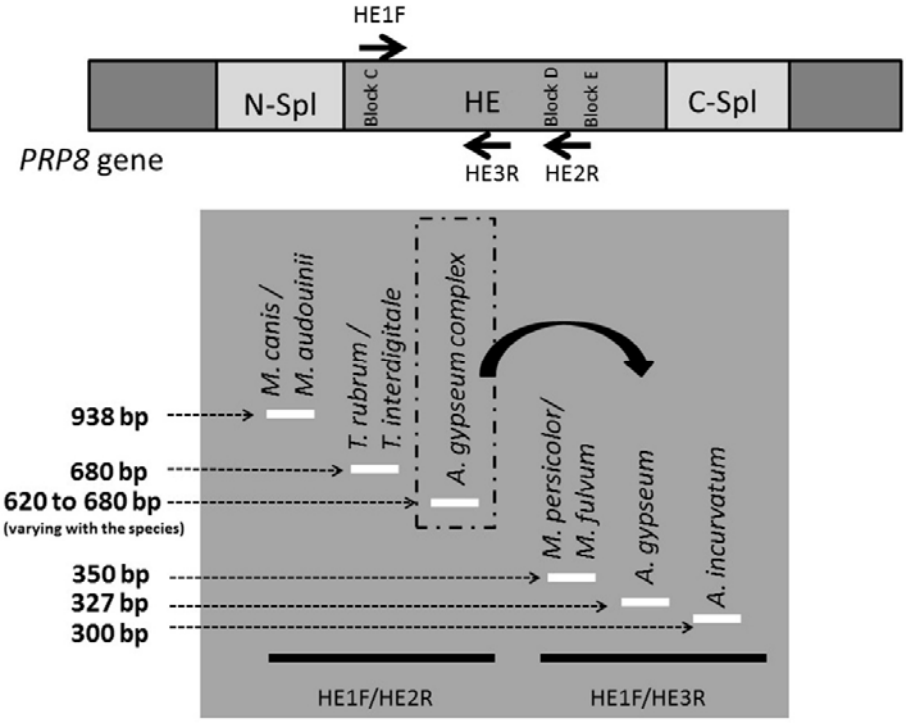

Figure 1: Proposed diagram to differentiate clinically important dermatophytes (Microsporum species), according to the PRP8 intein (HE domain) length polymorphism. Primers HE1F and HE2R anneal into Block C and Blocks D / E of the HE region, respectively. The first assay, using HE1F and HE2R primers, differentiates M. canis / M. audouinii (938 bp) from the other dermatophyte species. The second, using HE1F and HE3R primers, differentiates within the A. gyspseum species complex.

Results.

Dermatophytes might be identified using the PRP8 intein sequences.

The PRP8 intein was efficiently amplified for all species and individuals; all of them are full-length inteins. Among the samples herein evaluated, *M. canis* and its closest species *M. audoinii* present the largest intein with 1686 bp, while *A. incurvatum* has the smallest with 1449 bp (Table 1). All species with previously deposited sequences of the PRP8 gene (complete genomes at WGS database) showed an identity of 100% or 99% with the query sequence and E value of 0.0, corroborating the previous identifications using nuclear ribosomal regions for the same strains [19].

The PRP8 intein protein sequences, which obviously are more conserved than their corresponding DNA sequences, showed 99% or 100% similarity to those previously deposited identified species (Data not shown). Interestingly, this was not observed for *M. canis,* whose PRP8 intein protein sequence (converted from the DNA sequence herein obtained) showed 98% similarity to the deposited PRP8 intein region of this species. Analyzing this result, we found a sequence of 11 aa corresponding to 33 nucleotides (just beside the Block E of the HE) within the intein that was not translated at the GenBank database for *Arthroderma otae* CBS 113480 (accession number XP_002846024). Therefore, we hypothesized that these 33 nucleotides might constitute an intron. In fact, all characteristics that define an intron were found in this region [56]. Considering that translation for this protein sequence is made by conceptual translation (theoretically) and that similar aligned sequences for this region are present in other remaining dermatophytes species with 100% identity when compared to query sequences, we carried out a RT-PCR assay in *M. canis* and *M. audouinii* for determining whether this putative intron really occurs, and may be spliced and, therefore, not expressed. Since the 33 nucleotide sequences were found in cDNA from the *PRP8* gene, for strains 747, 830 and 483, we concluded that this is not a functional intron and in this case is normally translated. Thus, the whole DNA sequence for *M. canis* and *M. audouinii* was translated and deposited in GenBank including this region.

Dermatophytes present a full-length PRP8 intein with an apparently active LAGLIDADG HE.

For all species, a full PRP8 intein with a HE belonging to LAGLIDADG family was found. The splicing domain showed no length polymorphism among the different dermatophytes. However, a significant length polymorphism was noticed between blocks C and D of the HE domain for different species. For dermatophytes herein studied, the length from Block C to Block D was 279 aa for *M. canis* and *M. audouinii*, 195 aa for *T.*

interdigitale, *Trichophyton tonsurans* and *T. rubrum*, 193 aa for *E. floccosum* and *Microsporum fulvum*, 189 aa for *A. gypseum*, 188 aa for *Trichophyton* ajelloi, 183 aa for *Microsporum persicolor* and 177 aa for *A. incurvatum* (Supplementary Material 1)

The analysis of dS/dN rates of conserved block of PRP8 intein provided systematically high values both for splicing and HE domains in all species herein evaluated (Supplementary Material 2 and 3) [49]. The dS/dN rates for HE conserved-block domains are nearly identical to the splicing conserved-block domains for all compared species, indicating that besides splicing activity, the HE might also be active in this fungal group. Higher rates were particularly observed for the geophilic species *T. ajelloi* and species of *A. gypseum* complex (*A. gypseum*, *A. incurvatum*, *M. fulvum* and *M. persicolor*) and for the anthropophilic species *E. floccosum,* both for splicing and HE domains. The zoophilic dermatophyte *M. canis* seems to have a lower dS/dN rate, but still strongly positive, suggesting HE activity.

The presence of two essential residues of aspartic acid was also determined within blocks C and E related to HE functioning [22, 57]. It was found that these two essential residues are present but a substitution of Asp by glutamic acid (Glu) is present in all *A. gypseum* complex species and *E. floccosum* into Block E (Supplementary Material 1).

Phylogenetical analyses of PRP8 intein sequences reflect evolution of dermatophytes.

Phylogenetic constructions employing DNA and protein sequences of PRP8 intein, carried out in a representative group of dermatophytes (40 strains,11 species), generated similar topologies, with high bootstrap values, confirming the robustness of the analyses (Figures 2 and 3). The following clades and/or species complex were well defined, as proposed by Gräser et al., (2008): i) *T. interdigitale* close to *T. tonsurans*, which belongs to the *Arthroderma vanbreugseghemii* complex; ii) *T. rubrum* as an isolated group; iii) *M. canis* close to *M. audouinii*, which belongs to the *A. otae* complex; iv) the *A. gypseum* complex, which groups *Microsporum gypseum* / *A. incurvatum* separately from *M. fulvum*, *M. gypseum*

/ *A. gypseum*, *M. persicolor*, and also *E. flocosum; v)* finally, *T. ajelloi* as an independent clade.

Clinically important *Microsporum* species can be differentiated by a PCR-electrophoresis assay,

Finally, as a practical application, taking advantage of the above mentioned polymorphism in the HE region, two PCR-electrophoresis assays were designed for distinguishing species among the genus *Microsporum*. The tested strains belong to species with proven clinical importance, related mostly to the *Microsporum* genus [1, 4, 10, 20, 58-60]. Figure 4 shows an electrophoresis for identifying *M.canis* / *M. audouinii* from other clinically important dermatophytes by using the primers HE1F and HE2R. The primer HE1F anneals in a region next to Block C whereas the HE2R primer anneals in a region between blocks D and E. Both were designed for amplifying all herein evaluated dermatophyte species. *M. canis* and *M. audouinii* were separated from other dermatophytes, with amplicons of about 930 bp, while *A. gypseum*, *A. incurvatum, M. persicolor* and *M. fulvum* showed amplicons of about 600 to 700 bp (Figure 4A). In addition, *T. rubrum* and *T. interdigitale* were slightly separated from *A. gypseum* and *A. incurvatum* with amplicons of about 680 bp (Figure 4B).

Figure 5 shows a second electrophoresis for differentiating species from the *A. gypseum* complex. A third reverse primer HE3R was designed using the HE1F as the forward primer. The primer HE3R anneals in a region between blocks C and D and it is specific for species of the *M. gypseum* complex herein evaluated. Strains belonging to *A. incurvatum* with amplicons of 300 bp were separated from strains of *A. gypseum* with amplicons of 327 bp. The last two species, *M. persicolor* and *M. fulvum*, were also evaluated, being separated from *A. gypseum* and *A. incurvatum,* showing amplicons of about 350 bp.

Discussion.

Our results indicate that PRP8 intein is suitable for species identification among dermatophytes. Intein sequences correctly identified, with accuracies equivalent to rDNA sequences, the species *M. canis*, *A. gypseum*, *T. interdigitale, T. tonsurans* and *T.rubrum* that present genome data already deposited at Genbank. Some sequences showed low similarity values (<99%) in relation to other species (different from their identification by rRNA regions) because their sequences for *PRP8* gene or PRP8 intein were not deposited in the GenBank database. Once additional PRP8 intein dermatophytes sequences are deposited in databases, further research studies might employ this genomic region for identifying dermatophyte species or to confirm previous identifications by ribosomal DNA regions.

The studied dermatophytes showed a full-length PRP8 intein with a LAGLIDADG HE. As previously mentioned, a significant polymorphism was found related to the HE domain. Butler et al. (2006), by comparing PRP8 inteins from different fungi, observed that domains between the conserved blocks C and D might range from 212 to 300 residues[22]. This polymorphism-length pattern was observed repeatedly in more than one isolate of the species *M. canis* (four isolates), *T. tonsurans* (two isolates), *A. incurvatum* (four isolates), *A. gypseum* (three isolates), *E. floccosum* (two isolates), *T. rubrum* (nine isolates), *T. interdigitale* (twelve isolates), indicating that this region reflects the species phylogeny and might be used as a molecular marker to differentiate them.

The HE region tends to be more polymorphic because it is actually not necessary for intein splicing, having no role in Prp8 protein function and therefore, in cell survival. Since this polymorphism is reflected in a phylogeny that corroborates the topology previously obtained for nuclear ribosomal regions [3, 19], we can infer that the PRP8 intein has evolved vertically among the dermatophytes, which means no lateral transfer was detected. Even for very close species, such as those within the *A. gypseum* complex, the PRP8 intein, due to HE

polymorphisms, was sufficiently informative for species distinction. In this way, *A. gypseum*, *A. incurvatum*, *M. persicolor*, and *M. fulvum* were completely distinguished in the PRP8 phylogeny.

During evolution, dermatophytes underwent adaptation to different hosts, passing from soil to humans and other animals [10, 12, 15]. This adaptation to new hosts led to changes in reproduction, with the geophilic dermatophytes being the species retaining sexual reproduction [16]. This flux of genes caused by sexual reproduction during the speciation process in geophilic dermatophytes could lead to a higher variability and polymorphism even within closer species (species of the *A. gypseum* complex). This is reflected by the accuracy of both markers, the rRNA region (sequence polymorphism) as well as PRP8 intein (sequence and size polymorphisms) (Supplementary Material 1) in distinguishing these species.

In relation to HE activity, the dS/dN and phylogenetic analyses suggest that the majority of the HE of the PRP8 inteins in ascomycetes are active. This suggestion contrasts with the activity of the HE of the VMA intein, which has been proven to be inactive, in most of the species [22, 61]. The dS/dN rate is a measure of the selective pressures acting on two compared gene sequences. Higher dS/dN rates imply an abundance of synonymous substitutions, which means that the encode protein is going through a structural and functional conservation. When dS/dN rates present very low or negative values it signifies an abundance of non-synonymous substitutions implying that the referred protein is degenerating. The ratio of non-synonymous to synonymous substitutions in a protein-coding gene reflects the relative influence of purifying, positive or neutral selection [49].

As the PRP8 gene is essential for mRNA splicing, the intein splicing domain must operate perfectly, to maintain a functional Prp8 protein[62]. So it is expected that dS/dN values for this domain are higher than those for the HE domain, even when it is active, because, as mentioned before, the HE is not crucial for intein splicing, and therefore extein functionality

[34, 57, 63]. It seems that the HE of dermatophytes remains active since their dS/dN rates are high and nearly identical to the domains of splicing conserved blocks (Supplementary Material 2 and 3). *E. floccosum*, despite being an anthropophilic dermatophyte, shows high values because it is a species related to *A. gypseum* complex species proved by phylogenetic constructions employing ITS and D1/D2 regions [19, 64] and confirmed by phylogenetic constructions using the PRP8 intein in the present work (Figure 2 and 3). The HE region for this dermatophyte may have been conserved during evolution from an ancestral species originating the *A. gypseum* species complex and *E. floccosum*. Butler et al. (2006) by employing the dS/dN rates determined that HEs of PRP8 inteins are mainly active[22]. However, Theodoro et al. (2011) found by applying the same methodology that the HE might not be active for *P. brasilensis*[24].

For the VMA intein, the importance of two aspartic acid residues (Asp) was demonstrated at the positions 218 and 326 for HE activity [57]. By aligning PRP8 intein sequences with the HE VMA intein, Butler et al. (2006) determined that two Asp residues located in the blocks C and E of the PRP8 intein HE are fundamental for its activity on account of finding that almost all PRP8-intein-containing species show these two aminoacids residues. Theodoro et al. (2011), analyzing the HE function of the PPR8 intein from *B. dermatitidis*, *E. parva* and four cryptic species of *P. brasiliensis*, found a substitution of the aspartic acid in place of a glycine in Block E in *P. brasiliensis* and *E. parva,* and a serine in *P. lutzii,* confirming previous results by the dS/dN method[24]. For species belonging to the *A. gypseum* complex and *E. floccosum*, a substitution of Asp for Glu was found in Block E. However, as both Asp and Glu are very similar amino acids (acids and polar), this substitution may not disrupt the HE function. We can conclude that the HE of the PRP8 intein in dermatophytes may be active given the high dS/dN rates within conserved blocks of the HE

domains and the presence of two Asp or Glu residues within blocks C and E. However, this *in silico* analysis cannot replace an experimental assay for HE activity.

Both phylogenetic constructions shown in figures 2 and 3 were consistent with other previous phylogenies by using other genomic regions. Cafarchia et al. (2012) and Gräser et al. (2008), by analyzing the phylogenetic relationships among dermatophytes using nuclear ribosomal regions (ITS region), found similar phylogenies but inconsistencies in relation to *Epidermophyton* genus clustering[3, 64]. Our previous phylogenetic constructions were also inconclusive as to *E. floccosum* clustering, depending on the nuclear ribosomal region used [19]. For ITS-5.8S-ITS2, E. *floccosum* clustered with *A. gypseum* complex, specifically with *M. persicolor*, but when using the D1/D2 region it clustered with the *T. rubrum* / *T. violaceum* complex. However, by joining both regions ITS1-5,8S-ITS2 and D1/D2, *E. floccosum* maintained the same clustering observed by employing the PRP8 intein (DNA and protein sequences), validating the PRP8 intein phylogenetic constructions (Figures 2 and 3). It seems that *E. floccosum* becomes anthropophilic starting from a species related to the *M. gypseum* complex or they share a common ancestor. An additional fact supporting this idea is the substitution pattern of the essential Asp for Glu within Block E of the HE that occurs only in species of *M. gypseum* complex and *E. floccosum*.

Another remarkable difference can be observed in relation to the *A. vanbreuseghemii* complex that encompass several species belonging to the genus *Trichophyton* such as *T. mentagrophytes*, *T. interdigitale*, *T benhamiae* and *T. tonsurans* among others. Gräser et al. (2008) based on a polyphasic study have proposed to group several former *T. mentagrophytes* complex species into the single species *T. interdigitale,* maintaining as *T. mentagrophytes* only the species *T. mentagrophytes* var *quinckeanum*[3]. However, a recent study conducted by De Hoog et al. (2017) proposed a new taxonomic classification by using a phylogenetic multilocus study and other characteristics by which they separated and recognized several *T.*

interdigitale as *T. mentagrophytes*[12]. Both authors considered *A. vanbreugseghemii* to be a separated species. Previous nuclear ribosomal phylogenies using the same strains of this work separated strain B19 (*T. interdigitale*, zoophilic) and strain 433 (*A. vanbreuseghemii*) [19], which according to recent nomenclature are classified as different species of *T. interdigitale* [12]. Phylogenetic analysis of the PRP8 intein does not support this species separation since strains B19 and 433 clustered together with remaining *T. interdigitale* strains. In fact, there is no nucleotide polymorphism among these strains in the PRP8 intein. However, the related species *T. tonsurans* were efficiently clustered as a separated species. Although, a speciation process could be occurring in *T. interdigitale* species, the PRP8 intein phylogeny supports the idea of grouping all *T. mentagrophytes* species into the single species *T. interdigitale* proposed by Gräser et al, (2008). [3]. Even the species *A. vanbreuseghemii* could be grouped into *T. interdigitale,* according to this particular genomic region.

T. rubrum from an African population (strain 1024), recognized nowadays as *T. soudanense* [12], was separated from other *T. rubrum* strains by D1/D2 rDNA [19], but using the PRP8 intein it was clustered among *T. rubrum* strains. The proposal made for Gräser et al., (2008) to consider *T. soudanense* as a single species within *T. rubrum* [3] is supported herein, since no differences were found when DNA of PRP8 intein sequences was analyzed for nucleotide changes.

M. canis and *M. audouinii* were efficiently separated showing results similar to those from ITS-5.8S-ITS2 and D1/D2 phylogenetic constructions [19]. Nucleotide changes were detected in *M. audouinii* PRP8 intein DNA sequences compared with *M. canis* (98% identity, Table 1). This finding suggests that *M. audoinii* and *M. canis* are supported as separate species. This fact validates PRP8 intein as a suitable additional marker for phylogenetic species recognition, in a multilocus approach. All remaining species were grouped according to ITS and D1/D2 rDNA genomic regions [19].

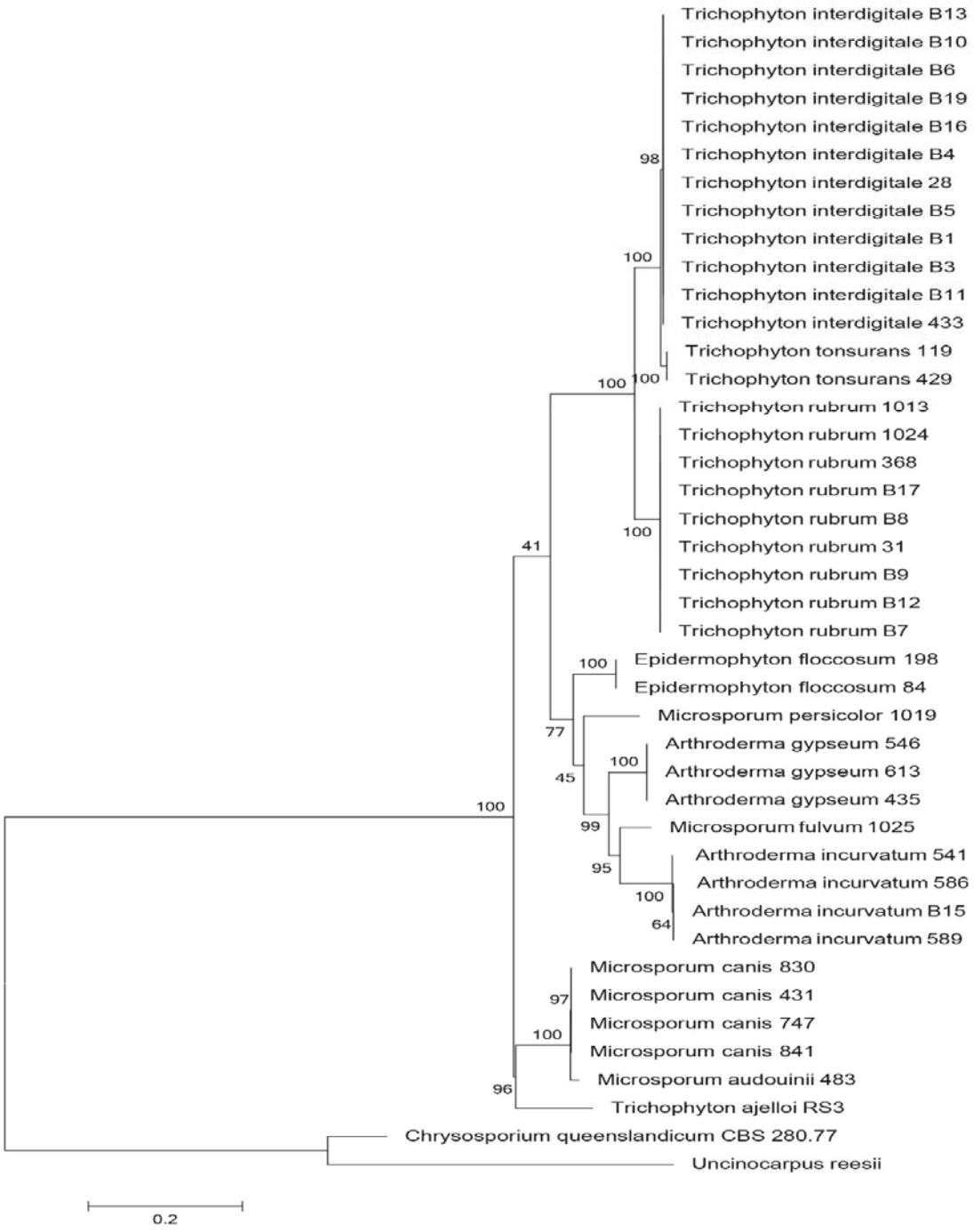

Figure 2: Phylogenetic construction using DNA sequences of the PRP8 intein in dermatophytes. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood is shown. The percentage of trees, in which the associated taxa are clustered together, is displayed next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 42 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were 1310 positions in the final dataset.

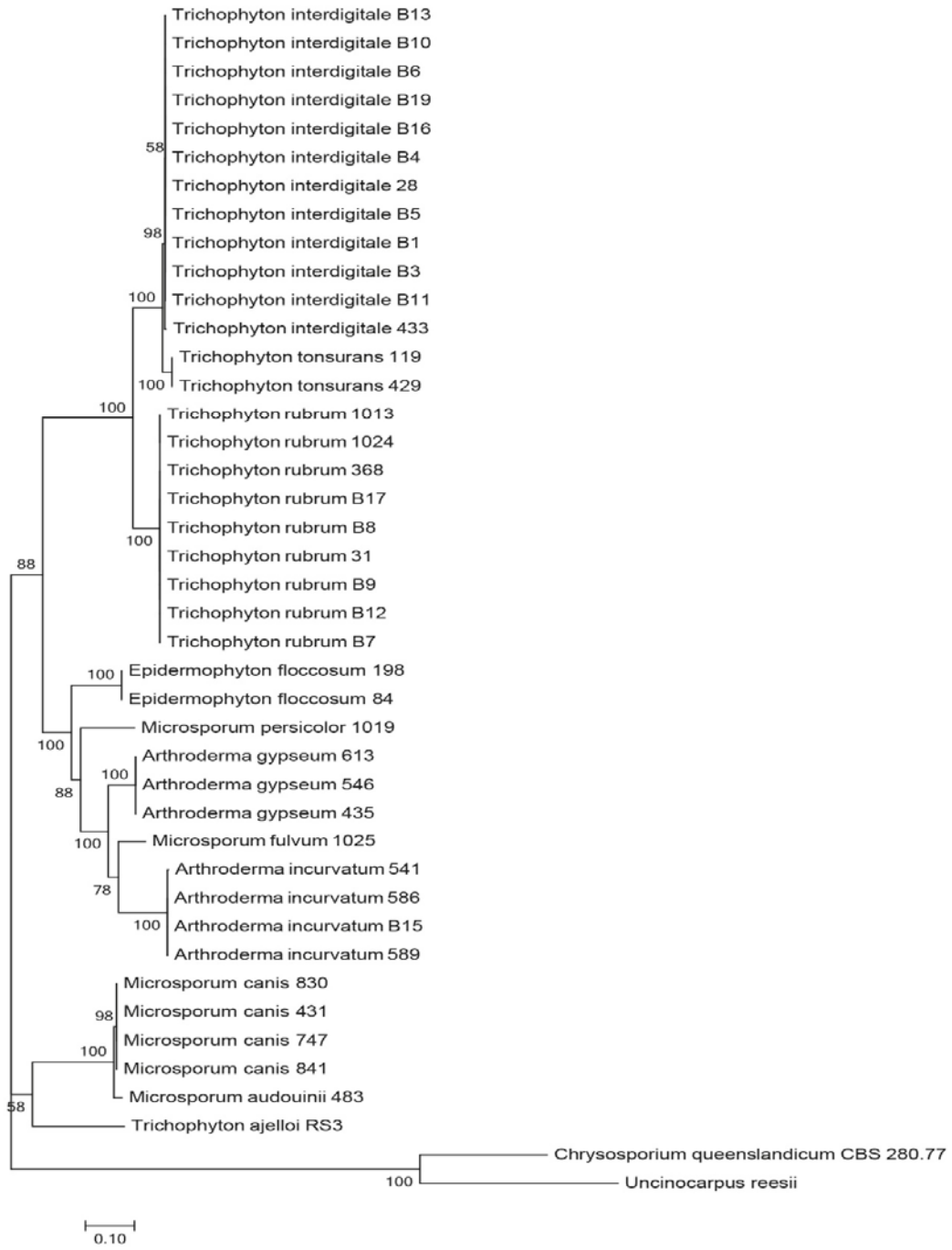

Figure 3: Phylogenetic construction using protein sequences of the PRP8 intein in dermatophytes. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood is shown. The percentage of trees, in which the associated taxa are clustered together, is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis involved 42 amino-acid sequences. There were 591 positions in the final dataset.

As a practical application, we attempted to design two assays for distinguishing medically important *Microsporum* species (Figure 1). The first PCR-electrophoresis assay can be used to identify *M. audouinii* and *M. canis* in a simple and accurate way with no need of sequencing. Although other dermatophytes herein sequenced and studied were not tested for amplification (ex: *E. floccosum*, *T. ajelloi* and *T. tonsurans*), it was proved by bioinformatics that amplicons must range around 650 bp for all three species, so that *M. audouinii* and *M. canis* can still be distinguished when these species are involved in the diagnosis.

As to the second procedure involving the *A. gypseum* species complex, we should remark that *A. gypseum* and *A. incurvatum* were considered distinct teleomorph states for the single anamorph specie *M. gypseum* [3]. Nowadays, *A. incurvatum* is considered a new species with its own teleomorph *Nannizia incurvata,* while *A. gypseum* was renamed as *N. gypsea* [12]. Despite being different species, they are not well differentiated morphologically, demanding molecular methods, such as sequencing of nuclear ribosomal regions, for their distinction [3]. This assay might represent an alternative to differentiate *A. gypseum* (*N. gypsea*) species from *A. incurvatum* (*N. incurvata*) when morphological traits are not sufficient.

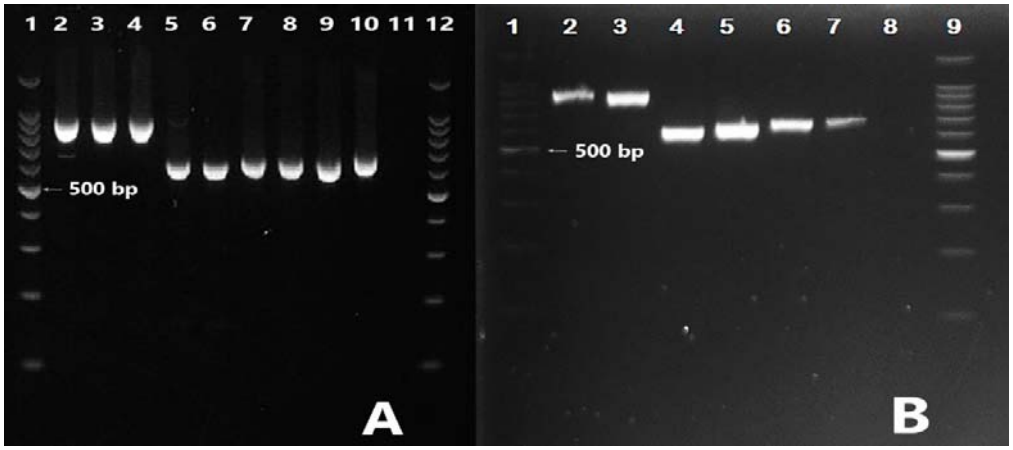

Figure 4: Electrophoresis of PCR products from PRP8 intein HE region for identifying *M. audouinii* and *M. canis*. **A**) HE1F/HE2R PCR products of *Microsporum* genus. Lanes 2 to 11 correspond to *M canis* (strain 747), *M. canis* (strain 841), *M. audouinii* (strain 483), *A. incurvatum* (strain 586), *A. incurvatum* (strain 589), *A. gypseum* (strain 546), *A. gypseum* (strain 613), *M. persicolor* (strain 1019), *M. fulvum* (strain 1025) and negative control, respectively. **B**) Electrophoresis of clinically important species of *Microsporum* and *Trichophyton* genus. Lane 2 to 8: correspond with *M. canis* (strain 841), *M. audouinii* (strain 483), *A. incurvatum* (strain 589), *A. gypseum* (strain 546), *T. rubrum* (strain 31), *T. interdigitale* (strain 28) and negative control, respectively. Lanes 1 and 12 of Figure 1 A and lanes 1 and 9 of Figure 1 B correspond with the 100 bp Molecular Marker (Thermo Scientific, Carlsbad, USA).

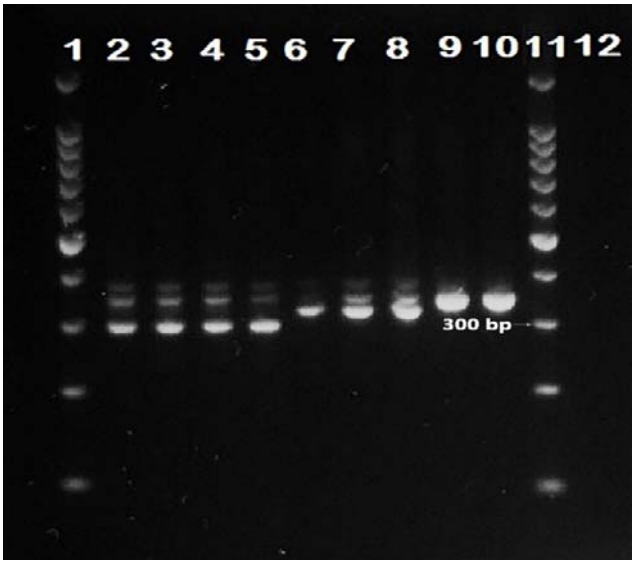

Figure 5: Electrophoresis of HE1F/HE3R PCR products from PRP8 intein HE for identifying *A.gypseum* complex species. Lanes 2 to 5 correspond to *A. incurvatum* (strains 589, 586, 541, B15); lanes 6 to 8 correspond to *A. gypseum* (strains 613, 435, 546); and lanes 9 and 10 correspond to *M. persicolor* (strain 1019) and *M. fulvum* (strain 1025), respectively. Lanes 1 and 11 contain the 100 bp Molecular Marker (Thermo Scientific, Carlsbad, USA) and lane 12 the negative control.

Conclusion.

This is the first study on the PRP8 intein in dermatophytes. All studied species present a full-length PRP8 intein, with a HE belonging to the family LAGLIDADG. PRP8 inteins are hereby proven to constitute an efficient molecular marker in order to determine the phylogenetic relations among dermatophytes. Further studies can be performed by using other species such as some members from the *A. vanbreuseghemii* complex and *T. violaceum* for elucidating species borderlines and taxonomy. Because of the polymorphism difference in the HE region, some *Microsporum* species can be easily identified by a PCR-electrophoresis assay, as herein proposed.

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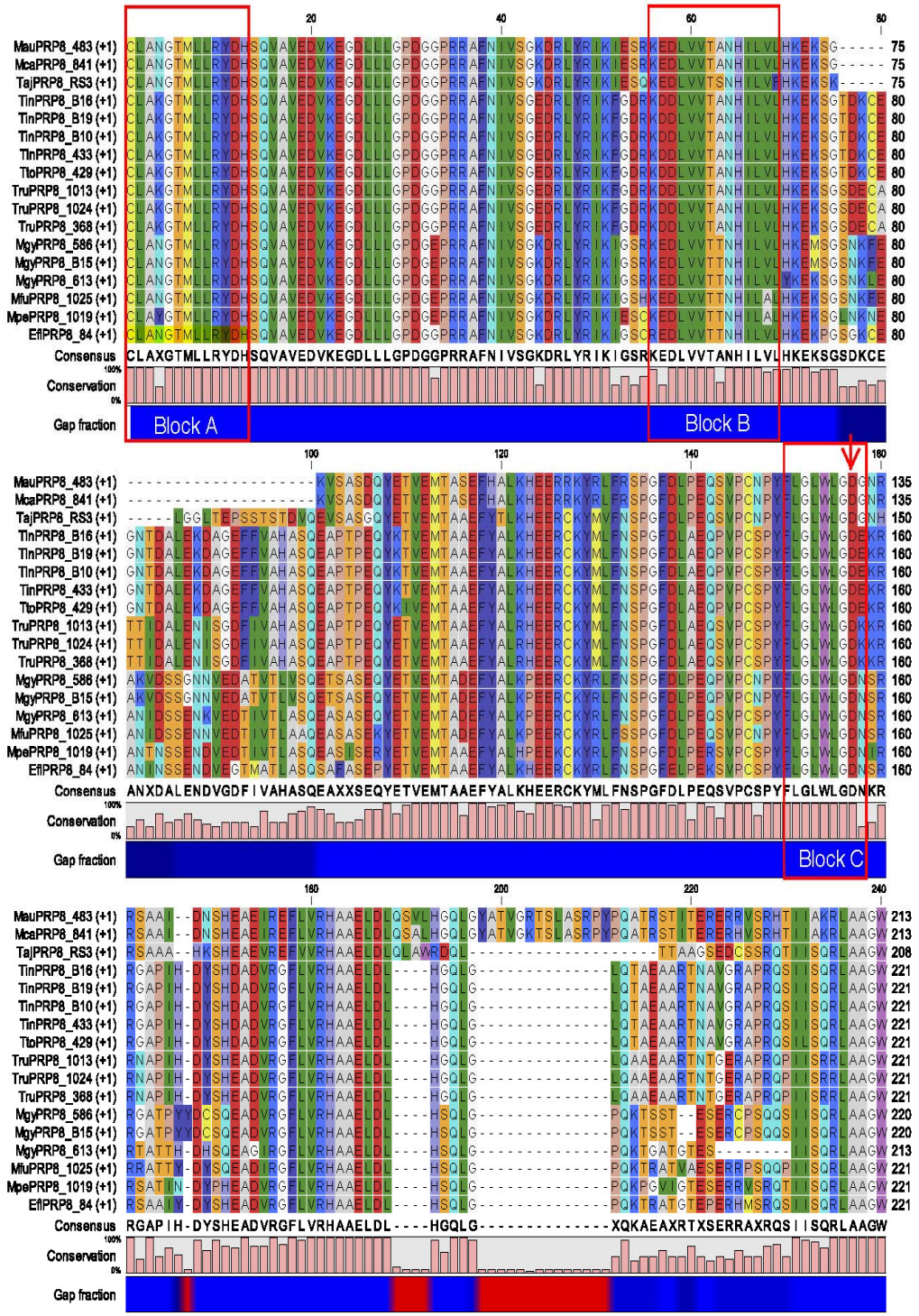
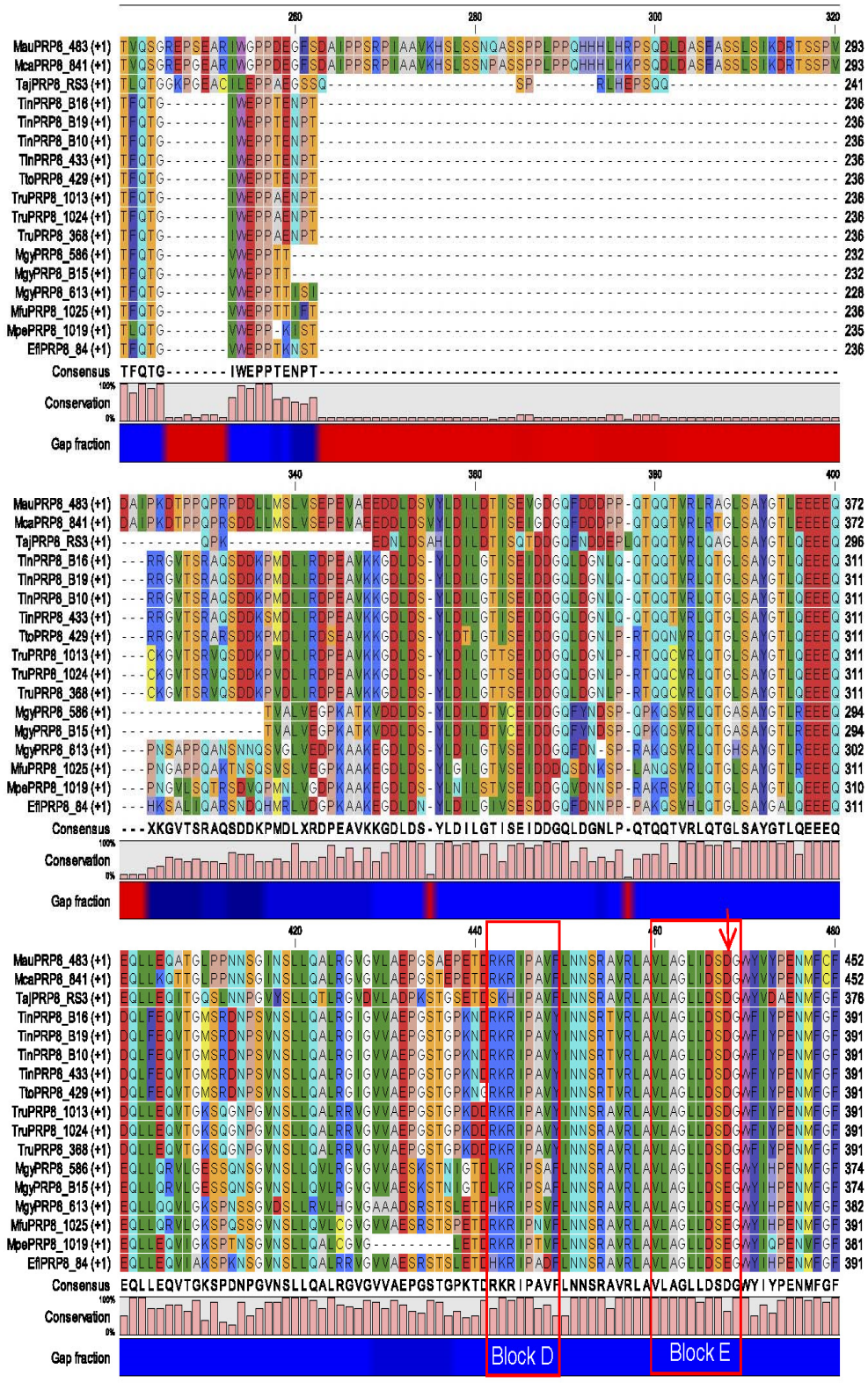

Block A
Block B
Block C
Consensus
Conservation
Gap fraction


MauPRP8_483 (+1)
McaPRP8_841 (+1)
TajPRP8_RS3 (+1)
TinPRP8_B16 (+1)
TinPRP8_B19 (+1)
TinPRP8_B10 (+1)
TlnPRP8_433 (+1)
TtoPRP8_429 (+1)
TruPRP8_1013 (+1)
TruPRP8_1024 (+1)
TruPRP8_368 (+1)
MgyPRP8_586 (+1)
MgyPRP8_B15 (+1)
MgyPRP8_613 (+1)
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Conservation
Gap fraction
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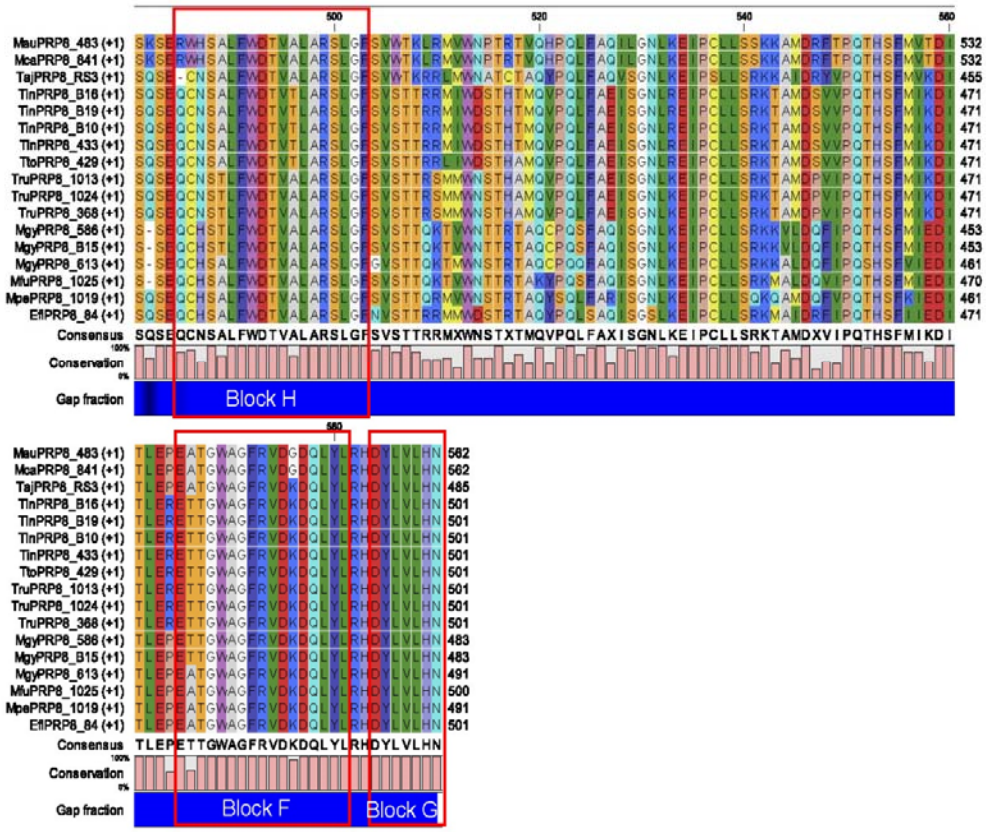

Supplementary material 1: Aligned full PRP8 intein for all dermatophyte studied species. Conserved block A, B, F and G belong to splicing domain while blocks C, D, E and H belong to HE domain. Mca: *Microsporum canis*, Mau: *Microsporum audouinii*, Taj: *Trichophyton ajelloi*, Tin: *Trichophyton interdigitale*, Tto: *Trichophyton tonsurans*, Tru: *Trichophyton rubrum*, Mgy: *Microsporum gypseum* (strain 586 and B15 belong to A. *incurvatum* and strain 613 to A. *gypseum*), Mfu: *Microsporum fulvum*, Mpe: *Microsporum persicolor*, Efl: *Epidermophyton floccosum*. Red arrows in Blocks C and E correspond to two essential residues for HE functioning (Aspartic or Glutamic acid).

Intein 1	Intein 2	Sd	Nd	S	N	pS	pN	dS	dN	dS\dN
Mca	Mau	0.00	0.00	36.00	115.00	0.00	0.00	0.00	0.00	-
Mca	Taj	9.00	4.00	35.17	115.83	0.26	0.03	0.31	0.04	8.85
Mca	Efl	2.00	4.00	35.83	115.17	0.06	0.03	0.06	0.04	1.63
Mca	Mgy/Ain	9.00	4.00	35.33	115.17	0.25	0.03	0.31	0.04	8.79
Mca	Mgy/Agy	6.00	3.00	35.67	115.33	0.17	0.03	0.19	0.03	7.20
Mca	Mpe	7.00	5.00	35.67	115.33	0.20	0.04	0.23	0.04	5.10
Mca	Mfu	9.00	4.00	35.83	115.17	0.25	0.03	0.31	0.04	8.60
Mca	Tru	5.00	6.00	35.83	115.17	0.14	0.05	0.15	0.05	2.86
Mca	Tin	12.00	10.00	71.33	79.67	0.17	0.13	0.19	0.14	1.39
Mca	Tto	6.00	5.00	35.67	115.33	0.17	0.04	0.19	0.04	4.27
Taj	Efl	11.00	3.00	35.00	116.00	0.31	0.03	0.41	0.03	15.48
Taj	Mgy/Ain	11.00	3.00	34.50	116.50	0.32	0.03	0.42	0.03	15.85
Taj	Mgy/Agy	10.00	2.00	34.83	116.17	0.29	0.02	0.36	0.02	20.78
Taj	Mpe	10.00	4.00	34.83	116.17	0.29	0.03	0.36	0.04	10.27
Taj	Mfu	17.00	3.00	35.00	116.00	0.49	0.03	0.78	0.03	29.72
Taj	Tru	9.00	6.00	35.00	116.00	0.26	0.05	0.31	0.05	5.88
Taj	Tin	22.00	10.00	69.67	81.33	0.32	0.12	0.41	0.13	3.05
Taj	Tto	11.00	5.00	34.83	116.17	0.32	0.04	0.41	0.04	9.25
Efl	Mgy/Ain	9.00	2.00	35.17	115.83	0.26	0.02	0.31	0.02	17.92
Efl	Mgy/Agy	8.00	1.00	35.50	115.50	0.23	0.01	0.27	0.01	30.78
Efl	Mpe	9.00	3.00	35.50	115.50	0.25	0.03	0.31	0.03	11.70
Efl	Mfu	9.00	2.00	35.67	115.33	0.25	0.02	0.31	0.02	17.53
Efl	Tru	5.00	6.00	35.67	115.33	0.14	0.05	0.16	0.05	2.88
Efl	Tin	12.00	10.00	71.00	80.00	0.17	0.13	0.19	0.14	1.40
Efl	Tto	6.00	5.00	35.50	115.50	0.17	0.04	0.19	0.04	4.29
Mgy/Ain	Mgy/Agy	7.00	1.00	35.00	116.00	0.20	0.01	0.23	0.01	26.83
Mgy/Ain	Mpe	10.00	3.00	35.00	116.00	0.29	0.03	0.36	0.03	13.67
Mgy/Ain	Mfu	10.00	2.00	35.17	115.83	0.28	0.02	0.36	0.02	20.47
Mgy/Ain	Tru	9.00	4.00	35.17	115.83	0.26	0.03	0.31	0.04	8.85
Mgy/Ain	Tin	20.00	6.00	70.00	81.00	0.29	0.07	0.36	0.08	4.61
Mgy/Ain	Tto	10.00	3.00	35.00	116.00	0.29	0.03	0.36	0.03	13.67
Mgy/Agy	Mpe	9.00	2.00	35.33	115.67	0.25	0.02	0.31	0.02	17.79
Mgy/Agy	Mfu	9.00	1.00	35.50	115.50	0.25	0.01	0.31	0.01	35.53
Mgy/Agy	Tru	8.00	5.00	35.50	115.50	0.23	0.04	0.27	0.04	6.01
Mgy/Agy	Tin	14.00	8.00	70.67	80.33	0.20	0.10	0.23	0.11	2.15
Mgy/Agy	Tto	7.00	4.00	35.33	115.67	0.20	0.03	0.23	0.04	6.50
Mpe	Mfu	14.00	1.00	35.50	115.50	0.39	0.01	0.56	0.01	64.26
Mpe	Tru	9.00	7.00	35.50	115.50	0.25	0.06	0.31	0.06	4.90
Mpe	Tin	20.00	12.00	70.67	80.33	0.28	0.15	0.36	0.17	2.13
Mpe	Tto	10.00	6.00	35.33	115.67	0.28	0.05	0.36	0.05	6.61
Mfu	Tru	10.00	6.00	35.67	115.33	0.28	0.05	0.35	0.05	6.51
Mfu	Tin	22.00	10.00	71.00	80.00	0.31	0.13	0.40	0.14	2.92
Mfu	Tto	11.00	5.00	35.50	115.50	0.31	0.04	0.40	0.04	8.96
Tru	Tin	8.00	0.00	71.00	80.00	0.11	0.00	0.12	0.00	-
Tru	Tto	4.00	0.00	35.50	115.50	0.11	0.00	0.12	0.00	-
Tin	Tto	0.00	0.00	70.67	80.33	0.00	0.00	0.00	0.00	-
Mean										11.85

Supplementary material 2

Table 1 supplemementary: Data describing nucleotide substitution patterns within the conserved regions of the splicing domains (A, B, F and G) of pairs of dermatophytes PRP8 inteins. Sd is the number of observed synonymous substitutions; Nd is the number of observed non-synonymous substitutions; S is the number of potential synonymous substitutions; N is the number of potential non synonymous substitutions; pS is the proportion of observed synonymous substitutions; pN is the proportion of observed non-synonymous substitutions; dS is the Jukes-Cantor correction for multiple hits of pS; dN is the Jukes-Cantor correction for multiple hits of pN. Intein for each species are identified by following (Mca: *M.canis*, Mau: *M. audouinii*, Taj: *T. ajelloi*, Efl: *E. floccosum*, Mgy/Ain: *M. gypseum*/*A. incurvatum*, Mgy/Agy: *M.gypseum*/*A. gypseum*, Mpe: *M. persicolor*, Mfu: *M. fulvum*, Tru: *T. rubrum*, Tin: *T. interdigitale*, Tto: *T. tonsurans*).

Intein 1	Intein 2	Sd	Nd	S	N	pS	pN	dS	dN	dS\dN
Mca	Mau	0.00	0.00	35.00	104.00	0.00	0.00	0.00	0.00	-
Mca	Taj	9.00	6.00	33.83	102.17	0.27	0.06	0.33	0.06	5.37
Mca	Efl	10.00	8.00	33.83	105.17	0.30	0.08	0.38	0.08	4.68
Mca	Mgy/Ain	14.50	11.50	33.83	105.17	0.43	0.11	0.64	0.12	5.38
Mca	Mgy/Agy	15.75	9.25	34.33	104.67	0.46	0.09	0.71	0.09	7.54
Mca	Mpe	7.75	7.25	34.83	104.17	0.22	0.07	0.26	0.07	3.61
Mca	Mfu	12.75	8.25	34.17	104.83	0.37	0.08	0.52	0.08	6.21
Mca	Tru	11.25	9.75	34.00	105.00	0.33	0.09	0.44	0.10	4.40
Mca	Tin	9.50	8.50	34.83	104.67	0.28	0.08	0.35	0.09	4.02
Mca	Tto	10.50	8.50	34.33	104.67	0.31	0.08	0.39	0.09	4.57
Taj	Efl	14.50	8.50	33.17	102.83	0.44	0.08	0.66	0.09	7.49
Taj	Mgy/Ain	19.50	11.50	33.17	102.83	0.59	0.11	1.15	0.12	9.49
Taj	Mgy/Agy	20.25	9.75	33.67	102.33	0.60	0.10	1.21	0.10	11.92
Taj	Mpe	12.75	8.25	34.17	101.83	0.37	0.08	0.52	0.09	6.02
Taj	Mfu	19.75	9.25	33.50	102.50	0.59	0.09	1.16	0.10	12.03
Taj	Tru	16.25	8.75	33.33	102.67	0.49	0.09	0.79	0.09	8.70
Taj	Tin	15.50	7.50	33.67	102.33	0.46	0.07	0.71	0.08	9.25
Taj	Tto	14.50	7.50	33.67	102.33	0.43	0.07	0.64	0.08	8.30
Efl	Mgy/Ain	14.75	5.25	32.67	106.33	0.45	0.05	0.69	0.05	13.53
Efl	Mgy/Agy	14.00	3.00	33.17	105.83	0.42	0.03	0.62	0.03	21.48
Efl	Mpe	7.00	3.00	33.67	105.33	0.21	0.03	0.24	0.03	8.39
Efl	Mfu	13.00	4.00	33.00	106.00	0.39	0.04	0.56	0.04	14.43
Efl	Tru	12.00	8.00	32.83	106.17	0.37	0.08	0.50	0.08	6.31
Efl	Tin	9.00	9.00	33.17	105.83	0.27	0.09	0.34	0.09	3.73
Efl	Tto	10.00	9.00	33.17	105.83	0.30	0.09	0.39	0.09	4.27
Mgy/Ain	Mgy/Agy	13.00	3.00	33.17	105.83	0.39	0.03	0.55	0.03	19.19
Mgy/Ain	Mpe	12.00	4.00	33.67	105.33	0.36	0.04	0.48	0.04	12.41
Mgy/Ain	Mfu	8.00	4.00	33.00	106.00	0.24	0.04	0.29	0.04	7.56
Mgy/Ain	Tru	13.00	9.00	32.83	106.7	0.40	0.08	0.56	0.09	6.26
Mgy/Ain	Tin	12.00	12.00	33.17	105.83	0.36	0.11	0.49	0.12	4.02
Mgy/Ain	Tto	13.00	12.00	33.17	105.83	0.39	0.11	0.55	0.12	4.51
Mgy/Agy	Mpe	11.00	2.00	34.17	104.83	0.32	0.02	0.42	0.02	21.77
Mgy/Agy	Mfu	9.00	2.00	33.50	105.50	0.27	0.02	0.33	0.02	17.32
Mgy/Agy	Tru	16.75	9.25	33.33	105.67	0.50	0.09	0.83	0.09	8.93
Mgy/Agy	Tin	13.75	10.25	33.67	105.33	0.41	0.10	0.59	0.10	5.66
Mgy/Agy	Tto	14.75	10.25	33.67	105.33	0.44	0.10	0.66	0.10	6.31
Mpe	Mfu	10.00	1.00	34.00	105.00	0.29	0.01	0.37	0.01	38.96
Mpe	Tru	9.00	7.00	33.83	105.17	0.27	0.07	0.33	0.07	4.71
Mpe	Tin	6.00	8.00	34.17	104.83	0.18	0.08	0.20	0.08	2.49
Mpe	Tto	7.00	8.00	34.17	104.83	0.20	0.08	0.24	0.08	2.97
Mfu	Tru	12.75	8.25	33.17	105.83	0.38	0.08	0.54	0.08	6.55
Mfu	Tin	11.75	9.25	33.50	105.50	0.35	0.09	0.47	0.09	5.07
Mfu	Tto	12.75	9.25	33.50	105.50	0.38	0.09	0.53	0.09	5.70
Tru	Tin	4.00	3.00	33.33	105.67	0.12	0.03	0.13	0.03	4.52
Tru	Tto	5.00	3.00	33.33	105.67	0.15	0.03	0.17	0.03	5.78
Tin	Tto	1.00	0.00	33.67	105.33	0.03	0.00	0.03	0.00	-
Mean										8.67

Supplementary material 3

Table 2 supplementary: Data describing nucleotide substitution patterns within the conserved regions of the homing endonuclease domains (C, D, E and H) of pairs of dermatophytes PRP8 inteins. Sd is the number of observed synonymous substitutions; Nd is the number of observed non-synonymous substitutions; S is the number of potential synonymous substitutions; N is the number of potential non synonymous substitutions; pS is the proportion of observed synonymous substitutions; pN is the proportion of observed non-synonymous substitutions; dS is the Jukes-Cantor correction for multiple hits of pS; dN is the Jukes-Cantor correction for multiple hits of pN. Inteins are identified using strain names as shown in Table 1 supplementary.